PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FERMENTED FOOD "MALEDA" OF NORTHERN INDIAN STATE

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Abstract
*The present study was focused to isolate lactic acid bacteria (LAB) from traditional fermented leftover dough 'maleda' Himachal Pradesh (India) to evaluate the probiotic potential. The assortment of samples was done from the different areas of the state. From the samples, 13 positive isolates showed the typical appearance of LAB. The screening of isolates was done through antibacterial activity against every test microorganism (*Staphylococcus aureus, Klebsiella pneumoniae *and* Escherichia coli*). Out of 13 isolates, maximum zone of inhibition was observed in* TS_6 *against* Escherichia coli *(0.6mm) and optimum was observed in case of* TM_9 *against* Staphylococcus aureus *(0.14mm) and* Escherichia coli *(0.15mm) where the least ZOI was noticed in TM1 isolate against* Staphylococcus aureus *(0.15mm) and* Escherichia coli *(0.2mm). Selected isolated bacterial cultures were optimizing for different environmental conditions including pH, bile salt, NaCl tolerance and bacteriocin production. Selected isolate* TS_6 *was able to grow on a wide range of Ph and exhibited excellent acid tolerance at pH 6, bile salt tolerance at 1.5 %, and the maximum NaCl tolerance is observed at 1.0 %. The characterization of selected isolate* TS_6 *was performed by morphological, microscopic examination, different biochemical tests. By performing gram staining of the selected isolate TS6, it was found to be gram-positive bacilli, occurring in colonies.*

Keywords: Probiotic, Lactic Acid Bacteria, Bile Tolerance, Bacteriocin

Received: 28.07.2020 Reviewed: 18.12.2020 Accepted: 11.01.2021

INTRODUCTION

In the late 19th century, microbiologists recognized supportive micro-flora in the gastrointestinal (GI) tracts of healthy individuals which are different from those found in unhealthy individuals. These micro-floras exert health-promoting influences towards human, and further acknowledged as probiotics which literally mean 'for life' (Parvez *et al.* 2006). The terms probiotics expound "live microbial feed supplement which beneficially affects the host by refining microbial equilibrium" (Aslam and Qazi, 2010).

Demands for food containing probiotics are increasing worldwide due to the incessant creation of research verification indicating their prospective health benefits to consumers. Probiotic bacteria may produce a variety of compounds, including organic acids (acetic acid, lactic acid), reuterin and bacteriocin. These organic acids not only subordinate the pH but also disrupt the growth of the pathogen (Tambekar *et al.* 2010). Majority of microorganisms used as probiotics belong to Lactic acid bacteria (LAB) and *Bifidobacteria*. From the last few decades, extensive research conducted on the potential of Lactic acid bacteria (LAB) towards human health and revealed that they produce enviable micro flora of the gastrointestinal tract (GIT) thus called 'generally regarded as safe' (Fijan. 2014). The group of LAB, *Lactobacillus* species are most commonly utilized as a group of microorganisms for their probable beneficiary properties as probiotics. The antagonistic action of such bacteria is well-known to hamper a large number of enteric and urinary pathogenic bacteria (Naderi *et al.* 2014). LAB is a part of human microbiota and widely used as starter cultures in the food industries. Probiotics have a wide range of applications including in the treatment of acute diarrhea (Sanders *et al.* 2013), probiotics also used in the reduction of lactose in dairy products through fermentation and the replication of the probiotics in the gastrointestinal tract, which releases lactase (Rolfe. 2000; Ramos *et al.*

2013). It has been suggested that several probiotics can reduce cholesterol in the gut, as well as produce metabolites that obstruct with its synthesis in the liver (Sharma *et al.* 2018). Probiotics are also used to prevent food allergy. The mechanisms by which probiotics exert their effects are scattered in the literature. Some studies reveal that these effects may engage by modifying gut pH, antagonizing pathogens through production of antimicrobial compounds, competing for pathogen binding and receptor sites, further for accessible nutrients and growth factors, stimulating and safety issues around probiotics are reviewed.

Recent scientific research has supported the significant role of probiotics as a part of a healthy diet for humans as well as for animals and maybe an opportunity to provide a secure, cost-effective, and 'natural' approach that adds a barrier against microbial infection. In the Indian subcontinent, production and utilization of traditional fermented food using local food crops and other natural resources are very frequent among citizens of Himalayas (Nehal. 2013). Traditional fermented beverages and foods are well-liked in the rural and tribal areas of Himachal Pradesh (India). The fermented foodstuffs those are exclusive to the tribal and rural belts of Himachal Pradesh (India) are *siddu, bhaturu, dosha, sepubari, seera, chang, daru, lugri and anguri* (Thakur and Bhalla, 2004).

Different researches revealed that probiotics possess antagonistic activities toward the growth of pathogenic bacteria. This antagonistic effect could be due to the production of inhibitory compounds such as bacteriocin, hydrogen peroxides, and reuterin (Kolida et al. 2006; Yu et al. 2013). Fermented products are associated with healthy edible microbes which are beneficial for mankind. The broad variety of traditional fermented food and beverages are produced and consumed worldwide. Nowadays, researchers show a great interest in exploring the probiotic potential from traditional fermented products as a source of new isolates. However, limited work is done on the isolation of *Lactobacillus* strains from the major traditional inoculum source 'malera' (previously fermented leftover dough) of Himachal Pradesh, India (South Asia). Therefore present study aimed to monitor the probiotic potential of the isolated *Lactobacillus* strains by assessing their lenience against at different (pH, bile salt, and NaCl) concentrations and bacteriocin production.

MATERIAL AND METHODS

Sample Collection

Samples of traditional fermented leftover dough 'maleda' were collected from different areas of the state Himachal Pradesh (India) in sterile containers and stored in the refrigerator for further studies.

Isolation of Lactic Acid Bacteria

For the isolation of lactic acid bacteria (LAB), serial dilution agar technique was used. 1 g of each sample was dissolved into 9 ml of distilled water and shake homogeneously. Serial dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} was made by 1 ml into 9 ml of water blanks. 0.1ml of each dilution was inoculated to MRS agar plates and incubated at 37 ℃ for 24 h. The plates were observed for the appearance of colonies and a number of colonies produced on each plate of different dilution were recovered (Hoque *et al.* 2010). For purification of bacteria, streak plate method was used. Plates were incubated at 37 ℃ for 24 - 48 h and transferred to MRS agar slants. Further, plates are incubated at 37 ℃ and then maintained in the refrigerator at 4 ℃ till further analysis.

Strains

The test strains *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* were obtained from the culture collection of the department of microbiology, Shoolini University, Solan, Himachal Pradesh (India).

Screening of Selected Isolate for Antimicrobial Activity

In MRS broth, isolates were inoculated and incubated at 37 ℃ for 24 - 48 h. After incubation, agar well diffusion method was used to test the antimicrobial activity (Flow dig. 1) as given per literature Mishra and Prasad, (2005). The supernatant of LAB isolates was tested against the *Escherichia coli*,

Staphylococcus aureus and *Klebsiella pneumonia.*

Evaluation of the Probiotic Potential

The selected isolates with good antimicrobial activity were screened and treated at different concentrations of NaCl, pH, and bile salt as per methods given by (Ramos *et al.* 2013; Guo et al. 2009; Vinderola and Reinheimer. 2003; Yu *et al.* 2013).

pH Tolerance

The selected isolates were inoculated into sterile MRS broth tubes of varying pH, i.e. 2.0, 3.0, 4.0, 5.0, 6.0 and 6.5.

Bile Salt Tolerance

To determine bile salt tolerance selected isolates were inoculated into sterile MRS broth with varying concentration of bile salt at 0.5 %, 1.0 %, 1.5 %, and 2.0 %.

NaCl Tolerance

To determine the probiotic potential selected isolate was inoculated into sterile MRS broth at different NaCl concentration 0.2 %, 0.5 %, 1.0 % and 2.0 %.

Bacteriocin Production

A loop full culture of the selected isolates were inoculated in 150 ml of MRS broth and incubated at 37 °C for 24 h. For the production of bacteriocin, cell-free supernatant was obtained by centrifugation at 7000 rpm at 4 °C for 15 min. The obtained supernatant was stored at 4 °C for further study.

Assay for Bacteriocin Activity

The activity of bacteriocin was checked based on antibacterial potential against pathogenic microorganisms which are commonly observed in gastro-intestinal tract infections (*Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*). The antimicrobial activity of the supernatant was determined by agar well diffusion method. The wells were made with the help of a puncher and loaded with 200 µL of bacteriocin upon MRS agar plates containing each of the pathogenic organisms followed by incubation at 37 °C for 24 h. After the incubation plates were checked for antibacterial activity as a zone of inhibition and diameter (mm) of zones was measured as per the method given by (Kumari *et al.* 2016).

Characterization of Selected Bacterial Isolates

The selected bacterial isolates were identified based on their morphological, microscopic and biochemical characterization (catalase, coagulase, indole, MR-VP, citrate utilization, nitrate reduction, urease) according to Bergey's manual of determinative bacteriology (Holt *et al.* 1994).

Statistical Analysis

The observations of quantitative estimation of parameters were taken in triplicate and presented in Mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Isolation of Lactic Acid Bacteria

From the samples of maleda, 13 positive isolates showed the typical appearance of lactic acid bacteria on MRS medium were randomly selected and assayed for physiological properties as tabulated in (Table 1).

Screening of Isolated Bacterial Cultures for Antimicrobial Activity

Isolates of bacterial cultures were inoculated to MRS broth incubated at 37 °C for 24 - 48 h. The selected isolates were screened based on antibacterial activity against every test microorganism. Out of 13 isolates (Figure 1), the maximum zone of inhibition was observed in case of TS_6 against *Escherichia coli* (0.6mm) and optimum zone of inhibition was observed in case of TM_9 against *Staphylococcus aureus* (0.14mm) and *Escherichia coli* (0.15mm). The least zone of inhibition was observed in the case of TM_1 isolate against *Staphylococcus aureus* (0.15mm) and *Escherichia coli* (0.2mm) as shown in (Figure 2). Pundir et al. (2013) isolate bacterial cultures and each of them was screened against eight human bacterial pathogenic strains including, (*Escherichia coli*, *Salmonella enteria*, *Salmonella typhi*, *Staphylococcus epidermis* and *Bacillus amyloliqufacians*. This antibacterial activity might be due to the production of acetic and lactic acids that lowered the pH of the medium or competition for nutrients, due to production of bacteriocin or antibacterial compounds (Tambekar *et al.* 2009).

Sr. No.	Isolates	Microorganism	Colony morphology
1.	TM_1	Bacteria	Circular, Flat, Smooth, Entire, Translucent, White
2.	TM_2	Bacteria	Circular, Flat, Smooth, Translucent, White
3.	TM_3	Bacteria	Circular, Raised, Undulate, Smooth, Translucent, White
4.	TM_4	Bacteria	Circular, Flat, Entire, Smooth, Opaque, White
5.	TM_5	Bacteria	Circular, Small, Flat, Entire, Smooth, Opaque, White
6.	TS_6	Bacteria	Circular, Raised, Entire, Smooth, Opaque, Yellow
7.	TS_7	Bacteria	Circular, Large, Raised, Undulate, Smooth, Opaque, White
8.	TS_8	Bacteria	Round, irregular, Raised, Entire, Smooth, Opaque, White
9.	TS_9	Bacteria	Circular, Raised, Entire, Smooth, Opaque, White
10.	TS_{10}	Bacteria	Round, small, Raised, Entire, Smooth, Opaque, White
11.	TS_{11}	Bacteria	Round, small, Flat, Entire, Smooth, Opaque, White
12.	TP_{12}	Bacteria	Round, small, flat, Entire, Smooth, Opaque, White
13.	TP_{13}	Bacteria	Round, flat, Entire, Smooth, Opaque, White

All the samples of malera were collected from different areas of the state Himachal Pradesh (India)

Table 1 Isolates with their colony morphology

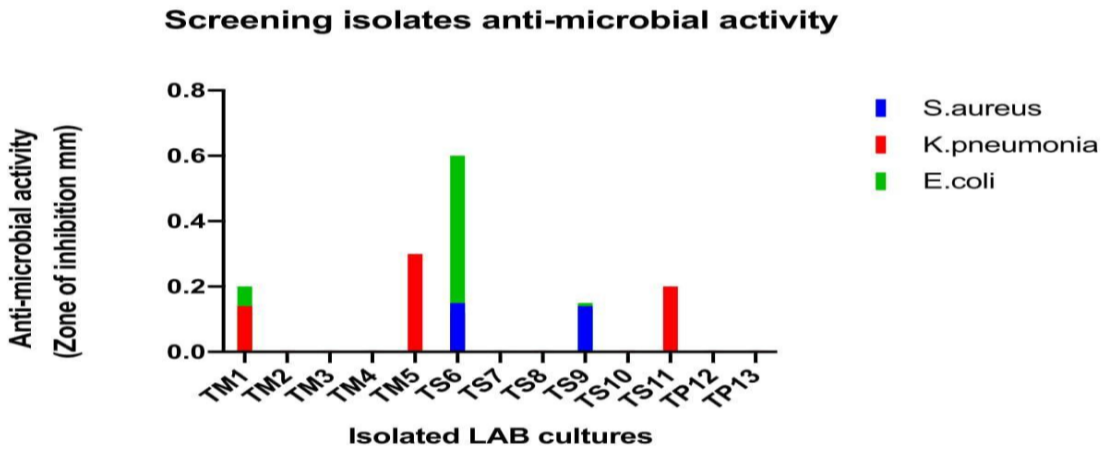

Figure 1. Antimicrobial activity of isolated LAB cultures against pathogenic strains by Agar well diffusion method

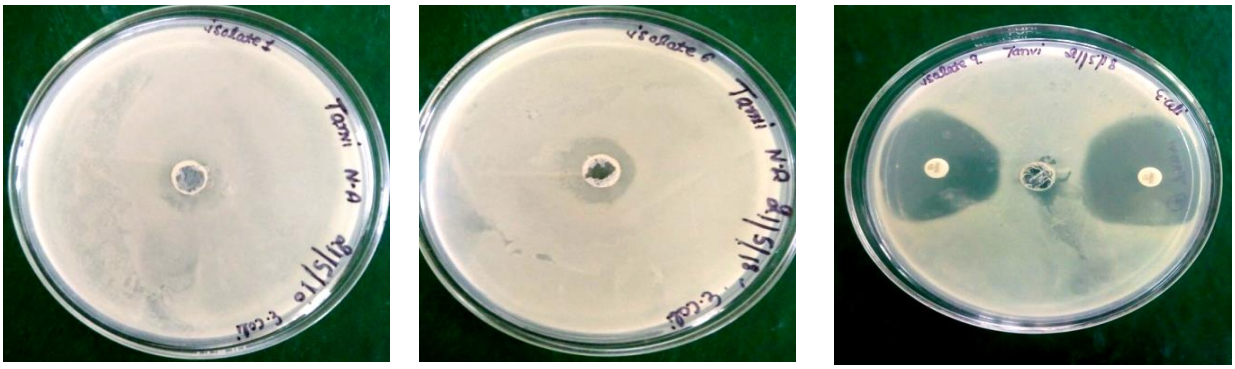

Figure 2 (a) Antimicrobial activity of TM1isolate against E. coli (b) Antimicrobial activity of TS6 isolate *E. coli* (c) Control with antibiotic discs

Optimization of Conditions for Selected Isolated Bacterial Cultures

Different environmental conditions including pH, bile salt, NaCl tolerance and bacteriocin production were checked for assessing maximum probiotic potential (Table 2).

pH Tolerance

To determine pH tolerance, isolated bacterial cultures were inoculated into sterile MRS broth tubes of varying pH 2.0, 3.0, 4.0, 5.0, 6.0 and 6.5 and incubated at 37 °C for 24 h. The selected isolate TS_6 was able to grow on a wide range of pH *viz.* 2.0, 3.0, 4.0, 5.0, 6.0 and 6.5 and it was observed that the isolate exhibited excellent probiotic acid tolerance at pH 6. Tambekar *et al.* 2010, reported that the *Lactobacillus* strains were viable after exposure to low pH and showed acid tolerance at pH 2 which favors the results obtained in the present study, whereas in another similar study *Lactobacillus* was capable to grow at low pH for 3 h (Kumari *et al.* 2016; Angmo *et al.* 2016). This will help bacteria to reach the small intestine and colon and contribute to balancing the intestinal micro flora (Tambkar *et al.* 2010).

Bile Salt Tolerance

Bile salts are produced in the liver, secreted into bile ducts and gallbladder, and sent from there to the small intestine by way of the common bile duct. In the intestine, it absorbs and digests the fat and fat-soluble vitamins. The selected LAB isolate was able to survive at 0.5 %, 1.0 %, 1.5 %, and 2.0 %. From the study it was observed that the isolated bacterial isolate has good bile salt tolerance at 1.5 %, which means it is essential for colonization and metabolic activity of bacteria. Similar results was obtained by (Tambekar *et al.* 2010), who grow the isolated cultures of *Lactobacillus* strains with varying bile salt concentration at 0.5 %, 1.0 %, 1.5 % and 2.0%. From this study they found that the *lactobacillus* strains were able to tolerate bile salt at 2.0 % concentration. Hassanzadazar *et al.* (2012), found the bile salt tolerance at 0.3 % in the isolates of *Lactobacilli* isolated from cheese.

NaCl Tolerance

NaCl is an inhibitory substance which may inhibit the growth of certain types of bacteria. In the present study, the selected bacterial isolates were treated with varying concentration of NaCl at 0.2 %, 0.5 %, 1.0 % and 2.0 %. Selected isolates were able to tolerate 0.2 %, 0.5 %, 1.0 %, and 2.0 % NaCl concentration. If the lactic acid bacteria were sensitive to NaCl then it would not be able to show its activity in the presence of NaCl. In the present study, the maximum NaCl tolerance is observed at 1.0 % NaCl concentration which means it can grow in the presence of alkali. Hoque *et al.* 2010, found the NaCl tolerance ranged at 1-9 % in the isolates of *Lactobacillus sp*. isolated from yoghurt.

Table 2. Effect of pH, bile salt and NaCl tolerance on selected isolate (TS_6)

pH						
pH	*2*	*3*	*4*	*5*	*6*	*6.5*
Dry cell Weight	*0.007±0.00*	*0.009±0.00*	*0.010±0.00*	*0.012±0.00*	*0.017±0.00*	*0.014±0.00*
Bile salt concentration %						
Concentration	*0.5*	*1.0*	*1.5*	*2.0*		
Dry cell Weight	*0.007±0.00*	*0.010±0.00*	*0.016±0.00*	*0.014±0.00*		
NaCl concentration %						
Concentration	*0.2*	*0.5*	*1.0*	*2.0*		
Dry cell Weight	*0.015±0.00*	*0.022±0.00*	*0.032±0.00*	*0.025±0.00*		

All the values are measured in triplicates and presented in mean ± SD, Dry cell Weight is measured in (g/L)

Figure 3. Plates showing antimicrobial activity against *E.coli*

Table 3 (a) Morphological characters of selected isolate TS_6

Identification	Isolate (TS_6)
Colony morphology on MRS agar	Rods (bacilli), yellow colonies
Gram's staining	Gram +ve bacilli (Purple color, Rod shape)

Table 3 (b) Biochemical tests of selected isolate TS_6

Biochemical analysis	Result
Catalase	-ve
Indole test	-ve
MR test	+ve
VP test	-ve
Citrate utilization test	-ve
Nitrate utilization test	+ve
Urease test	+ve
Motility	NM
Glucose	A/G
Lactose	A/G
Mannitol	A/G
Sucrose	A/G

A/G=Acid-gas production, NM=Non-motile, (+ve) indicates positive, (-ve) indicates negative

Production of Bacteriocin and Antimicrobial Activity

Selected isolate TS_6 was inoculated into 250 ml flask and incubated at 37 °C for 48 h, in an incubator shaker with sealed plugs. The maximum zone of inhibition was observed against *E. coli* (0.4mm) whereas there is no ZOI (Figure 3) in case of *S.aureus* and *K.pneumoniae* it means the isolated bacteria can produce bacteriocin and it may help to prevent various infections caused by *E. coli* such as bloody diarrhea, stomach cramps and mild dehydration etc. Kumari *et al.* (2016) obtained comparable results where five isolates were able to produce bacteriocin against different food-borne pathogens and spoilage bacteria. All five isolates exhibited antimicrobial activity against *E. coli* (with inhibition of 8.0-14.00 mm in diameter), *S. aureus* (9.2-16.3mm), *B. subtilis* (11.2-17.3) and *S. dysenteriae* (10.1-13.4mm). The possible mechanisms of bactericidal action include diminished pH levels, competition for substances, and the production of substances with a bactericidal or bacteriostatic action including bacteriocin and bacteriocin- like substances (Pan *et al.* 2009).

Characterization of the Selected Isolate

Characterization of the selected isolate TS_6 was performed by various methods *viz.* morphological, microscopic examination, and different biochemical tests (catalase, coagulase, indole, MR-VP, citrate utilization, nitrate

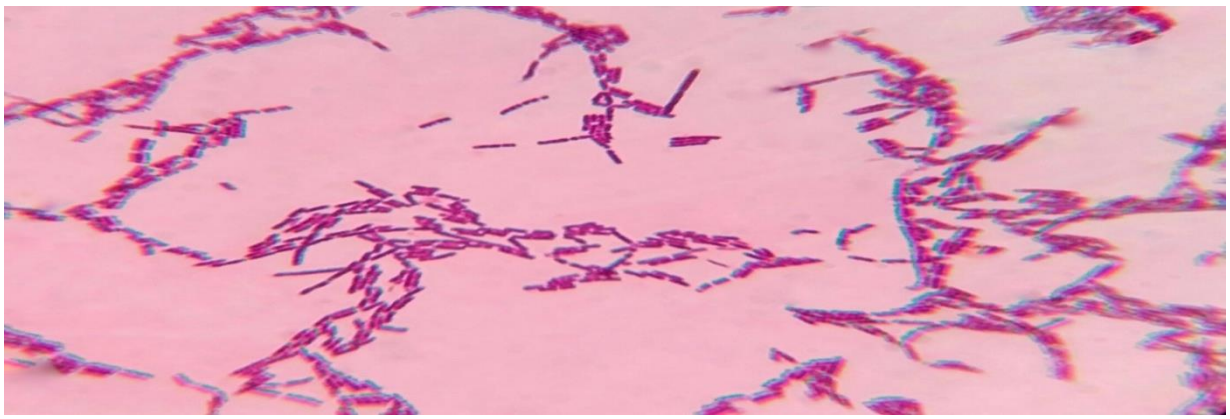

Figure 4. Microscopic view of TS_6

reduction, urease) was done to find out the source for growth and development. In the present study, isolate TS_6 showed better probiotic character and was identified on the basis of its morphological and biochemical characteristics are presented in Table 3 (a) and (b).

Morphological and Microscopic Examination

After the gram staining of TS_6, it was observed under a light microscope for morphological analysis. From the gram staining of the selected isolate TS_6, it was found to be Gram-positive bacilli, occurring in colonies (Figure 4).

CONCLUSION

Isolated *Lactobacillus* species are obtained from fermented foods samples of Himachal Pradesh (India) like maleda possess probiotic properties. A total of 13 isolates were obtained to check probiotic potential. Out of all the selected isolates, TS_6 isolates exhibit maximum probiotic potential by its ability to tolerate low pH, NaCl tolerance, and growth in the presence of bile salt which can prove it as a promising alternative in nutraceuticals. The selected isolate also produced bacteriocin and was tested for antimicrobial activity by performing agar well diffusion method then, it showed a maximum zone of inhibition against *E. coli*. These characteristics may be advantageous for a probiotic culture to be successful in colonizing in the human gut to produce desirable micro flora of the GIT tract and to reduce the incidence of intestinal infections.

ACKNOWLEDGMENT

The support by School of Biotechnology, Shoolini Institute of Life Sciences & Business Management, Solan, Himachal Pradesh, India is thankfully acknowledged.

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