Original Article

DEVELOPMENT OF IN VITRO METHODOLOGIES FOR INHIBITION OF PATHOGENIC BACTERIA BY POTENTIAL PROBIOTIC LACTOBACILLUS SPS; AN EVIDENCE FOR PRODUCTION OF ANTIMICROBIAL SUBSTANCES

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ABSTRACT

Objective: Probiotic products consist of specific strains of live bacteria that have potentially favorable health effects. A number of studies provide evidence that milk products with probiotics may be beneficial for digestive health and may improve various digestive problems. The purpose of the present study was to investigate Lactobacillus’s species with potential activities isolated from different cheese samples of local market.

Methods: A total 42 lactic acid bacteria strains were isolated, fourteen (14/42) best Lactobacillus isolates were selected by preliminary screening as potential probiotics with antimicrobial activity against pathogenic bacteria. All the fourteen Lactobacillus isolates were then characterized in vitro for their probiotic features and antimicrobial activities against pathogens.

Results: The results noticed that all selected Lactobacillus isolates (CH3, CH4 and CH6) were screened and confirmed as Lactobacillus. The isolates were able to grow at different pH, NaCl and bile salts, also exhibited the best antimicrobial activities against pathogens. All the isolates were susceptible to antibiotics used and isolates were also revealed the noticeable aggregation and hydrophobicity studies.

Conclusion: Selected Lactobacillus isolates were considered as ideal, effective probiotic bacteria. Thus, they could be examined further and contribute to preventing and controlling several infections associated with intestine and for human health benefits.

Keywords: Cheese, Antimicrobial activity, Antibiotic susceptibility test, Lactic acid bacteria, Probiotics

INTRODUCTION

The term probiotic was defined as “a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance” [1]. Probiotic bacteria may produce various compounds, which are inhibitory to the pathogen’s growth, which include organic acids (lactic and acetic acids), bacteriocins, and reuterin. The organic acids not only lower the pH, thereby affecting the growth of the pathogen, but they can also be toxic to the microbes [2]. There is increasing evidence that probiotics are beneficial in gastrointestinal disturbances, such as diarrhea, dysentery, typhoid etc [2]. It is important to underline when considering the effectiveness and biological activity of probiotics, prebiotics or their combination (symbiotic) that they are food products and not drugs.

The concept of using live bacterial species such as Lactobacillus sps with health benefit has received a great deal of attention in recent years. It is well known that the gastrointestinal (GI) tract is the home to a vast number of bacterial species, with vital roles in maintaining GI functionality including up to 70% of the immune system activity [3]. The probiotics are recommended as a preventive approach to maintain the balance of intestinal microbiota [4]. Amongst various microbiota, Lactobacillus sps is especially important for the maintenance of the human intestinal microbial ecosystem [5] which, in turn, may affect the quality of life. It has been indicated that the disturbances in the normal microbiota of the GI tract may lead to dysbiosis and ultimately clinical disease expression [6].

Lactobacillus sps that have wide spread use in fermented food production [7] and are considered as generally recognised as safe organisms and can be safely used for medical and veterinary applications [8]. In food industry, Lactobacillus sps are widely used as starter cultures and has been cited to be part of human microbiota [9, 10]. In raw milk and dairy products such as cheese, yoghurts and fermented milks, Lactobacilli are naturally present or added internationally, for technological reasons or to generate a health benefit for the consumer [11] and cheese is one of the best-known foods that contain probiotics [12]. From the health point of view, ingestion of live cells of certain species and strains the probiotic concept of Lactobacillus in adequate amounts is believed to confer several beneficial physiological effects on the host [13]. The criteria for the in vitro selection of Lactobacilli to be used as health-promoting, probiotic ingredients, in food and pharmaceutical preparations include antibiotic tolerance as well as the production of lactic acid that inhibits the growth of other microorganisms, which allow them to be established in the intestinal tract [14]. Bile tolerance [15] and gastric juice resistance [16] are other important characteristics of probiotic lactobacillus sps used as adjuncts because they enable them to survive, to grow and to perform their beneficial action in the gastrointestinal tract.

In the Gulbarga district and its surrounding region of Karnataka state, dairy products; cheese is possibly the oldest fermented milk product known and consumed by large sectors of the population as a part of their daily diet. In most of the urban areas of Karnataka state, different types of traditional cheese are found, but their probiotic role was not studied. Fusion of probiotic microorganisms (isolated from primitive cheese) in market of cheese can positively enhance health status of longer segment of communities. So this study is aimed to isolate the effective Lactobacillus isolates isolated from different products of cheese available at milk parlours and to determine the in vitro probiotic properties such as pH, NaCl tolerance, bile, antibiotic susceptibility profile, antimicrobial activity, aggregation studies and cell-surface hydrophobicity capacity of potentially selected probiotic Lactobacillus sps were demonstrated in controlling the growth of pathogenic strains.

MATERIALS AND METHODS

Material

Samples and other materials used in this research obtained from different companies of cheese, which are commercially available at
Collection of samples

Due to their high association with health benefits dates among the consumers of Gulbarga and its surrounding region of Karnataka state, the dairy products; cheese samples were collected randomly from different companies from retailers in the market of Gulbarga. The samples were transferred in transport media (stored at 4 °C) within 1 hr to the laboratory for microbiological analysis and processed within 24 h, further stored aseptically in low refrigerator temperature to protect normal flora and avoid from contamination.

Preliminary screening

In order to rapidly isolate acid and bile resistant bacteria from the plenteous microflora of cheese samples, the preliminary screening in phosphate buffer solution with pH 3.0 and 6.0 was performed for 3-6 h.

Isolation of Lactobacillus sps

The bacteria Lactobacillus sps were isolated from cheese by using MRS (de Man, Rogosa and Sharpe) medium. Each sample containing Ten gram of cheese was homogenized with sterile phosphate buffer solution (2% w/v) at 30-40 °C in a stomacher 200-400 circulator (Remi make, India). Then a volume of 2 ml of each dilution was added into 20 ml MRS broth (pH 6.5) and incubated at 37 °C for 24 h. Finally, the single colony of bacteria was observed by isolating their colony morphology and some biochemical tests (Gram staining, catalase and oxidase and motility test) and the culture were maintained in MRS (Obtained from Hi-media Pvt. Ltd, Bangalore) broth at 6.5 [17].

Identification of Lactobacillus sps

The isolated bacteria were identified as Lactobacillus sps by observing their morphological characteristics and by means Gram staining, motility, catalase, oxidase test and milk coagulation activities. The confirmed Lactobacillus isolates were further preserved at MRS broth with skim milk (10%) and glycerol (30%) in-20 °C. At last complimentary of isolated Lactobacillus isolates was determined with some standard Lactobacillus sps.

Optical growth at different pH

For the determination of the optimal growth at different pH of Lactobacillus isolates, a single isolated colony was subcultured in MRS broth, from that 1% (v/v) fresh overnight culture of Lactobacillus isolates was inoculated to varying pH between 2.5-7, adjusted to different pH using NaOH (1.0M) or HCl (1.0M) and incubated at 37 °C for 24 h. At last, each pH value, the visible color of the media were recorded and observed their turbidity at 600 nm.

Bile salt tolerance

The capability of strains to tolerate bile salts was determined according to the modified method of Gillibond and colleagues [19]. Lactobacillus isolates were tested for prompt growth in MRS broth medium with and without the addition of bile salts. MRS broth was prepared with the different concentration of bile salts between 0.5-2.5% added to 5 ml of the test tube and sterilized at 121 °C for 15 min. 0.1 ml of Lactobacillus isolates were inoculated, and bacterial growth was monitored by measuring absorbance at 600 nm after incubation for 18-24 h at 37 °C.

Assessment for NaCl tolerance

For the determination of NaCl tolerance, MRS broth containing different NaCl concentration between 1-6% was sterilized, each test tube was inoculated with 1% (v/v) fresh overnight culture of Lactobacillus and incubated at 37 °C for 24 h. After the incubation, their growth was determined by observing their turbidity at 600 nm.

Antibiotic susceptibility test

According to the standard working procedures, antibiotic susceptibility tests were done on Mueller-Hinton agar (Hi-media Pvt Ltd, India) using Kirby-Bauer disk diffusion method [20]. Various antibiotic with different classes were used; tetracycline (30 µg), ampicillin (30 µg), ethromycin (15 µg), chloramphenicol (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), cefadroxil (30 µg), ceftriaxone (30 µg), amoxicillin (10 µg), amoxiclav (10 µg), clindamycin (30 µg), neomycin (10 µg) and sulfamethoxazole (10 µg) (Hi-media, India). Resistance and sensitivity pattern data were interpreted according to National Committee for Clinical Laboratory Standards [21]. Reference strains of Lactobacillus fermentum NCDC 141 and Lactobacillus rhamnosus NCDC 329 (National Collection of Dairy Cultures, Karnal, India) were used for quality control for antibiotic susceptibility tests.

Antimicrobial (antagonistic) activity of Lactobacillus isolates

Antimicrobial activity of all collected Lactobacillus isolates against test pathogens were determined by agar-well diffusion method according to Ashral et al. [22]. Staphylococcus aureus (MTCC 96), Escherichia coli (MTCC 439), Klebsiella pneumonia (MTCC 432), Pseudomonas aeruginosa (MTCC 7925), E. coli (MTCC 443), Salmonella typhi (MTCC 7374), and Shigella spp (MTCC 13313) acquired from Microbial type collection culture Chandigarh, India, were used as test pathogens. A volume of 50-100 µl of the cell-free supernatant of each Lactobacillus isolates was filled in 7 mm diameter well in the nutrient agar including the test pathogens. The diameter of the clear inhibition zone was measured after 24 h of incubation. Each experiment was performed in triplicate.

Characterisation of antimicrobial substances

The characterisation of selected probiotic Lactobacillus isolates (CBH, CH4 and CH6) were evaluated for the production of antimicrobial substances like bacteriocins, organic acids and hydrogen peroxide using agar well diffusion technique with the slight modification described by Toure et al. [23]. The 25 ml of grown culture in MRS broth was divided into an equal portion for different assays. For Bacteriocin assay, 5 ml of supernatant treated with 1 mg/ml pronase or 1 mg/ml trypsin. For Organic acids assay, 5 ml of supernatant was adjusted to pH 6.5±0.1 using 1N NaOH and for hydrogen peroxide assay, 5 ml of supernatant was treated with 0.5 mg/ml catalase (Hi-media Pvt ltd). Treated supernatant were filtered with 0.2 µm pore size filters (Axiva pvt ltd) for bacteriocin assay. A volume of 50-100 µl was placed in 7-mm diameter wells; the plates were swabbed with 1% (v/v) overnight culture of each test pathogens. The inhibitory features were observed and measured the zone of inhibition after 24 h at 37 °C.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined to evaluate the phenotypic antimicrobial resistance of a strain to a certain probiotic Lactobacillus isolates (Cell-free culture supernatant). MIC was defined as the lowest Lactobacillus sps concentration that resulted in no visible growth. This MIC test was determined by broth dilution technique by following the reference standard established by CLSI 2010. Serial two-fold dilutions (Higher and lower) of the CFCS Lactobacillus isolates were inoculated with an overnight culture at a final concentration of 10^6 to 10^10 colony forming a unit (CFU/ml). MIC level was determined by measuring the test pathogen's absorbance at 600 nm and Lactobacillus-free broth used as a control.

Auto aggregation of probiotic Lactobacillus sps

Aggregation study was examined for effectively selected probiotic Lactobacillus isolates from cheese samples on the basis of their deposition properties. 18-24 h of fresh overnight cultures of each Lactobacillus isolates (10^6CFU/ml) were harvested by centrifugation at 5000g for 20 min, 4 °C, washed twice with Phosphate Buffer Saline (pH 7.2) and discarded in the same buffer. The auto aggregation percentage was calculated for three different Lactobacillus isolates after the mixture (vortexed) was incubated at 37 °C for 4 h without agitation.
Coaggregation of *Lactobacillus* sps with different test pathogenic cells

The co-aggregation study was examined for all three selected *Lactobacillus* isolates and different test pathogens according to a slight modified method to Collado et al. [24]. Bacterial cultures were separately cultured at 37 °C for 24 hours in MRS and Tryptic Soya Broth (TSB). Bacterial suspension (10⁸ CFU/ml) were prepared as narrated in the auto-aggregation as above method, an equal volume of cells of the different probiotic *Lactobacillus* sps and test pathogenic strains (1:1 v/v) were mixed and incubated at 37 °C without agitation. Absorbance, A₆₀₀ of the mixture illustrate above, was conducted during the incubation at 4 h, percentage of coaggregation were calculated as:

\[
\text{Coaggregation} \% = \frac{(A_{\text{pathogen}} + A_{\text{lactobacillus}})}{2 - A_{\text{mix}}} \times 100
\]

Where, Aₚₐ₉₉ and Aₙ₉₉₉₉ and Aₐ₉₉₉₉ represent the A₆₀₀ of the individual pathogen, *Lactobacillus* sps and their mixture after incubation for 4 h, respectively.

Time-kill assays with cell-free culture supernatant (CFCS) of *Lactobacillus* sps on test pathogens

The time–kill assay was performed by co-culture of each pathogenic cells and Cell-free culture supernatant (CFCS) of *Lactobacillus* sps, 300 µl of pathogenic suspension (10⁸ CFU/ml) were added into 15 ml of CFCS of each different *Lactobacillus* isolates, CFCS adjusted to be pH and MRS broth to 6.5 respectively and were incubated at 37 °C. At initial and designed/planned intervals, fractions were separated by serially diluting and plated on Luria-Bertani (LB) agar to determine the surviving cells of individual pathogens.

Cell surface hydrophobicity

Cell surface hydrophobicity was revealed, following to the capability of the three different *Lactobacillus* sps and test pathogens to partition into xylene from PBS [25] individually. The cells were washed twice with PBS and the optical density (A) at 540 nm adjusted to 0.5±0.01 to 1.0 ml of bacterial suspension, 60 µl xylenes was added and vortexed for 1 min and the optical density of the water phase was determined. Percentage of hydrophobicity was calculated according to the formula.

\[1 - \frac{(A_{\text{after}} - A_{\text{before}})}{A_{\text{before}}} \times 100\]

Quantification of organic acid and determination of pH value

One percent (v/v) 24 h active culture of *Lactobacillus* isolates was used to inoculate 10% sterilized skim milk (Hi-media pvt ltd India) and initial pH 6.76 was determined by digital electrode pH meter. The inoculated skim milk was incubated at 37 °C for 72 h and samples were collected in every 12, 24, 48 and 72 h and liquids of coagulated milk were separated by filtration. pH of the separated liquid was recorded using a digital electrode pH meter and quantification organic acid was performed through titration with 0.1N NaOH.

RESULTS

Isolation and identification of *Lactobacillus* isolate

Over the study period, a total five different companies of cheese samples collected from commercially available at local milk vendors of the city market. *Lactobacillus* sps were isolated from 14 (50%) of the total twenty-eight bacterial strains, among the collected bacterial strains, *Lactobacillus* sps were predominantly isolated (table 1).

| Source of dairy product | No. of isolates | No. of *Lactobacillus* sps |
|-------------------------|----------------|---------------------------|
| Amul cheese             | 13             | 5                         |
| Mozzarella pizza cheese | 8              | 3                         |
| Nilgiris Processed cheese | 5              | -                         |
| Nilgiris cheddar cheese  | 9              | 4                         |
| Milky mist cheese       | 7              | 2                         |
| Total                   | 42             | 14 (33.33%)               |

14 strains (after culturing for 48h), were selected as forming wide and white colonies on the selective MRS agar plate (fig. 1). Further, identified as *Lactobacillus* sps by observing their colony morphology, physiological as well as biochemical characterization (table 2). All the results clear that, bacteria were gram positive, rod shaped (fig. 2), non-motile and catalase negative. The confirmed *Lactobacillus* isolates were named as CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9, CH10, CH11, CH12, CH13 and CH14. These isolates were cultured on MRS with glycerol (30%) broth and stored at 20 °C.

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Table 1: Origin and number of isolates after screening for *Lactobacillus* sps

![Typical characteristics of the *Lactobacillus* isolates grown on MRS agar medium. (A) Isolated *Lactobacillus* colonies and (B) Single screened colonies on MRS media](image)
different bile salt concentration of each populations as depicted in the fig. 4, optical density values against Lactobacillus 0.05 to 2.5% of bile salt, at this bile concentration, all the samples shown maximum growth of the isolated Lactobacillus samples (OD=2.980), as shown in the fig. 3.

Bile salt tolerance

Five different companies of cheese samples (CH) collected from commercially available local milk vendors of the city market, Gulbarga, Karnataka (Number of isolated Lactobacillus sps from cheese samples: n=14).

| Selected Lactobacillus spp. | Morphological and cultural characteristics | Gram’s staining | Motility test | Catalase test | Carbohydrate fermentation test |
|-----------------------------|--------------------------------------------|----------------|--------------|--------------|--------------------------------|
| CH1                         | Small, irregular, smooth and circular      | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH2                         | Small, 0.1-0.5 mm, circular and round      | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH3                         | 1 mm, White, shiny smooth, round           | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH4                         | Shiny, Small Circular, white creamy        | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH5                         | 1.0 mm white, rough, irregular and round   | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH6                         | Small Circular, colourles s                | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH7                         | Small, 0.1-0.5 mm, rough dull and round    | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH8                         | 1.0 mm white, rough, irregular and round   | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH9                         | Small Circular, white creamy               | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH10                        | Small, 0.1-0.5 mm, rough dull and round    | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH11                        | Small Circular, white creamy               | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH12                        | 1.0 mm white, rough, irregular and round   | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH13                        | Small, 0.1-0.5 mm, rough dull and round    | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |
| CH14                        | 0.1-0.5 mm, white, irregular, smooth       | Gram+ve, bacilli | Non motile   | Negative     | -ve                            |

Five different companies of cheese samples (CH) collected from commercially available local milk vendors of the city market, Gulbarga, Karnataka (Number of isolated Lactobacillus sps from cheese samples: n=14).

Fig. 2: Microscopic observation of Gram’s stained Lactobacillus sps (Magnified at 100X)

Optical growth at different pH

All the isolated Lactobacillus sps of different sources of cheese samples shown maximum growth of the Lactobacilli isolated from Nilgiris cheddar cheese and mozzarella cheese was observed at pH5.0 to 6.5. The OD reading was the average value of the two samples (OD=2.980), as shown in the fig. 3.

Bile salt tolerance

The isolated Lactobacillus sps were capable of grow and survive in 0.05 to 2.5% of bile salt, at this bile concentration, all the Lactobacillus isolates were shown prompt multiplication in their populations as depicted in the fig. 4, optical density values against different bile salt concentration of each Lactobacillus isolates.

Fig. 3: Optimal growth and pH of isolated Lactobacillus isolates from Cheese samples, where CH-isolated Lactobacillus sps from cheese samples (n=14) at different pH (Error bars were omitted for simple and clear presentation)

Fig. 4: Bile acid tolerance of Lactobacillus isolates from Cheese samples, where CH-survival of isolated Lactobacillus sps from cheese samples (n=14) in 0.05 to 2.5% of bile salt (Error bars were omitted for simple and clear presentation)
NaCl tolerance test

All the selected Lactobacillus spp from different cheese samples were able to tolerate different NaCl concentrations i.e. 1-6%, results as shown in the fig. 5.

Antibiotic susceptibility test

The antibiotic susceptibility test was carried out for all 14 positive Lactobacillus spp against the 16 antibiotics consisted of different classes. Maximum lactobacillus isolates 10 (71.42%) were shown resistance to the antibiotic; ampicillin, 11 (78.57%) sensitivity to the antibiotic amoxiclav and 5 (35.71%) intermediate to the antibiotic erythromycin, the results as shown in the fig. 6 and table 3.

Antimicrobial (antagonistic) activity of Lactobacillus isolates

Antimicrobial activity is one of the main features of probiotic bacteria. For this reason, all the fourteen Lactobacillus isolates were examined for their potential inhibitory effects against different test pathogenic organisms (included gram positive and gram negative bacteria) such as Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumonia, Pseudomonas aerogenosa, Escherichia coli, Salmonella typhii and Shigella spp. using modified agar-well diffusion method. The results exhibited that all the isolates of Lactobacillus revealed the average inhibition (10-24 mm) on the growth of test pathogen, but the Lactobacillus isolates like CH3, CH4 and CH6 was the most effective noticeable isolates in inhibiting the growth of test pathogens (17-24 mm) than the reference strains of Lactobacillus fermentum NCDC 141 (fig. 7 and table 4a).

Further, Lactobacillus isolates were subjected for bacteriostatic or bacteriocidal, this confirmation test was done by modified agar overlaid method were conducted, swabs were taken from each clear zone of the test organism and were streaked onto the nutrient agar for growth. Based on the growth bacteriostatic and bacteriocidal activities are exhibited in table 4b. The presence of growth of test pathogen was confirmed as an inhibitory activity called bacteriostatic if no growth concludes as bacteriocidal.

Table 3: Antibiotic susceptibility test for Lactobacillus isolates

| S. No. | Antibiotics Used | No. of resistance | No. of sensitive | No. of intermediate |
|-------|-----------------|------------------|-----------------|-------------------|
| 1     | Ampicillin      | 10 (71.42%)      | 4 (28.57%)      |                   |
| 2     | Amikacin        | 7 (50%)          | 7 (50%)         |                   |
| 3     | Amoxyclov       | 2 (14.29%)       | 11 (78.57%)     | 2 (14.28%)        |
| 4     | Azthromycin     | 9 (64.28%)       | 4 (25.00%)      | 1 (7.14%)         |
| 5     | Ceftriaxone     | 10 (71.42%)      | 6 (42.85%)      | 1 (7.14%)         |
| 6     | Chlramphenicol  | 8 (57.14%)       | 6 (42.85%)      | --                |
| 7     | Ciproflouxacin  | 6 (42.85%)       | 8 (57.14%)      | --                |
| 8     | Cindamycin      | 8 (57.14%)       | 6 (42.85%)      | --                |
| 9     | Cotrimoxazol    | 5 (35.71%)       | 9 (64.28%)      | --                |
| 10    | Erythromycin    | 5 (35.71%)       | 4 (28.57%)      | 5 (35.71%)        |
| 11    | Gentamycin      | 6 (42.85%)       | 8 (57.14%)      | --                |
| 12    | Neomycin        | 6 (42.85%)       | 8 (57.14%)      | --                |
| 13    | Novobiocin      | 7 (50.00%)       | 7 (50.00%)      | --                |
| 14    | Streptomycin    | 7 (50.00%)       | 7 (50.00%)      | --                |
| 15    | Sulfamethizole  | 7 (50.00%)       | 7 (50.00%)      | --                |
| 16    | Tetracycline    | 5 (35.71%)       | 8 (57.14%)      | 1 (7.14%)         |

Among the 14 positive Lactobacillus spp, 10 (71.42%) Lactobacillus isolates were shown resistance to the antibiotic; ampicillin, 11 (78.57%) sensitivity to the antibiotic amoxiclav and 5 (35.71%) intermediate to erythromycin.
Fig. 7: Antimicrobial (antagonistic) activity of Lactobacillus isolates against different test pathogens, A. Gram-positive B. Gram-negative

Table 4a: Antagonistic activity of Lactobacillus isolates against test pathogens from Cheese samples

| Lactobacillus isolates | Zone of Inhibition in mm (from outer edge of Lactobacillus colony to outer edge of clear zone) |
|------------------------|------------------------------------------------------------------------------------------------|
|                        | S. aureus | S. typhi | E. coli | E. faecalis | K. pneumoniae | P. aerogenosa | Shigella spp. |
| CH1                    | 11        | 12       | 11      | 10          | 12            | 10           | 11          |
| CH2                    | 12        | 12       | 10      | 11          | 11            | 13           | 12          |
| CH3                    | 18        | 19       | 17      | 16          | 22            | 19           | 19          |
| CH4                    | 20        | 16       | 14      | 14          | 20            | 16           | 16          |
| CH5                    | 14        | 14       | 14      | 13          | 17            | 13           | 14          |
| CH6                    | 24        | 17       | 19      | 20          | 20            | 17           | 18          |
| CH7                    | 10        | 12       | 10      | 11          | 12            | 11           | 13          |
| CH8                    | 17        | 14       | 17      | 12          | 16            | 16           | 16          |
| CH9                    | 16        | 15       | 14      | 13          | 17            | 16           | 13          |
| CH10                   | 16        | 14       | 11      | 14          | 19            | 12           | 11          |
| CH11                   | 12        | 11       | 13      | 12          | 11            | 10           | 12          |
| CH12                   | 11        | 10       | 11      | 11          | 10            | 11           | 13          |
| CH13                   | 10        | 11       | 12      | 12          | 12            | 12           | 14          |
| CH14                   | 15        | 12       | 10      | 12          | 11            | 11           | 12          |

Isolates of Lactobacillus shown the average inhibition (10-24 mm) on the growth of test pathogen. CH3, CH4 and CH6 was the most effective noticeable isolates in inhibiting the growth of test pathogens (17-24 mm). All the Lactobacillus isolates were shown the average inhibition activity (10-24 mm). Where, CH3, CH1, and CH4 isolates showed effective Antagonistic activity (17-24 mm) against different test pathogens

Table 4b: Bacteriostatic and bactericidal activity of cheese isolates (CH3, CH4 and CH6)

| Name of the pathogens | CH3 isolate | CH4 isolate | CH6 isolate |
|-----------------------|-------------|-------------|-------------|
| S. aureus             | -           | -           | +           |
| E. faecalis           | +           | +           | -           |
| E. coli               | -           | -           | -           |
| P. aerogenosa         | -           | +           | +           |
| K. pneumoniae         | +           | -           | +           |
| S. typhi              | +           | +           | -           |
| Shigella spp.         | -           | -           | -           |

Where, + = Bacteriostatic, - = Bacteriocidal

Characterization of inhibitory substances

The effectively collected Lactobacillus isolates (CH3, CH4 and CH6) were evaluated for the characterization of inhibitory substances like bacteriocin, organic acid and hydrogen peroxide. This test was characterized by agar well diffusion assay against test pathogens. The results exhibited that culture supernatant of all three Lactobacillus sps and there reference strains treated with pronase (1 mg/ml) or trypsin (1 mg/ml) did not have any inhibitory activities effects of the Lactobacillus sps. This confirms that inhibitory effect of Lactobacillus isolates was due to bacteriocin production. Culture supernatants treated with catalase also did not affect the inhibitory activities of the Lactobacillus strains against the test pathogens. This showed that inhibition by the Lactobacillus strains was not due to hydrogen peroxides production. However, neutralized supernatant (pH 6.5) of all three Lactobacillus strains did not have any inhibitory activity effects of the Lactobacillus strains were due to their organic acid production. Hence, this study concludes that among three Lactobacillus isolates CH3 isolate was bacteriocin and CH4, CH6 isolates were responsible for organic acid production respectfully (fig. 8 and table 5).
Fig. 8: Antimicrobial activity of Lactobacillus (CFCS) Inhibitory substances against test pathogen, (A). Bacteriocin (B). Organic acid

Table 5: Characterization of antimicrobial substances of selected cheese isolates

| Shigella selected Strains | CH3 (in mm) | CH4 (in mm) | CH6 (in mm) |
|---------------------------|-------------|-------------|-------------|
| Bacteriocin assay         | Organic acid assay | Bacteriocin assay | Organic acid assay | Bacteriocin assay | Organic acid assay |
| S. aureus                 | 23          | -           | 16          | -           | 12          |
| E. faecalis               | -           | 17          | -           | 12          | -           |
| E. coli                   | 20          | -           | 19          | -           | 17          |
| P. aerogenosa             | -           | 21          | -           | 14          | -           |
| K. pneumoniae             | -           | -           | 11          | -           | 15          |
| S. typhi                  | 18          | 19          | 10          | -           | 19          |
| Shigella spp              | 19          | 15          | 16          | -           | 19          |

Neutralized supernatant of (pH 6.5) of all three Lactobacillus strains did not have any inhibitory activity effects. Among three Lactobacillus isolates CH3 isolate was bacteriocin and CH4, CH6 isolates were responsible for organic acid production. All the selected Lactobacillus isolates were subjected for the production of antimicrobial substances, CH4 and CH6 isolates were responsible for the production of only Bacteriocin, whereas CH3 isolate shown the production for both Bacteriocin and Organic acid against test pathogens.

Determination of minimal inhibitory concentration

All three Lactobacillus isolates were used for MIC test, the results clear that MIC for CH3 isolate was 50 µl against E. faecalis, S. typhi, S. aureus, k. pneumoniae, E. coli and p. aerogenosa, 100 µl for Shigella spp, for CH4 isolate 75 µl for k. pneumoniae, E. coli, p. aerogenosa and 100 µl for E. faecalis, S. typhi, S. aureus and Shigella spp and for CH6 isolate 128 µl for S. typhi, S. aureus, Shigella spp, E. faecalis and 100 µl for k. pneumoniae, E. coli, p. aerogenosa.

Auto and co-aggregation of probiotic Lactobacillus spp

The autoaggregation study was investigated for all three Lactobacillus isolates and different test pathogens based on their deposition capacity. The results exhibited that, among the three, CH3 isolate promptly noticed the highest percentage of auto aggregation after 24 h of the incubation period (51%) as compared to CH4 and CH6 isolates as shown in the table 6a.

The coaggregation results of the Lactobacillus isolates tested with different test pathogens as shown in the table 6b. This study is strain-specific as compared to aggregation, among the isolates, CH3, CH4 and CH6 isolates, showed the effective coaggregation with Shigella spp as 19.3, 17.4 and 20.4% respectively, similarly CH4 isolate showed the less coaggregation abilities with S. aureus, also the other test pathogens used.

Table 6a: Percentage of autoaggregation of Probiotic selected isolates with pathogenic strains

| Lactobacillus Strains | Auto-aggregation (%) |
|-----------------------|----------------------|
|                       | 4h | 18h | 24h |
| CH3 isolate           | 21±2.3 | 29±3.4 | 47±2.8 |
| CH4 isolate           | 14±2.5 | 21±2.5 | 38±2.7 |
| CH6 isolate           | 11±1.1 | 27±1.2 | 45±1.7 |
| Pathogenic strains    |     |     |     |
| Staphylococcus aureus | 2.9±1.0 | 3.8±0.1 | 5.0±0.9 |
| Enterococcus faecalis | 2.2±1.4 | 2.9±0.4 | 3.9±0.8 |
| Escherichia coli      | 7.2±1.2 | 12.1±0.8 | 16±1.2 |
| Pseudomonas aerogenosa| 3.5±0.8 | 11.1±1.1 | 20±1.5 |
| Klebsiella pneumoniae | 5.1±1.1 | 11±1.3 | 17±1.1 |
| Salmonella typhi      | 2.8±0.8 | 10±0.9 | 19±0.1 |
| Shigella spp          | 2.1±0.4 | 9.7±0.1 | 20±1.0 |

Each value is expressed in mean±SD (n=6 in each test group). Auto-aggregation is expressed in terms of Percentage (%) at 4, 18 and 24 h.

Table 6b: Percentage of coaggregation of Probiotic selected isolates with pathogenic strains

| Lactobacillus Strains | Co-aggregation (%) |
|-----------------------|--------------------|
|                       | 4h | 18h | 24h |
| CH3 isolate           |     |     |     |
| CH4 isolate           |     |     |     |
| CH6 isolate           |     |     |     |
| Pathogenic strains    |     |     |     |
| Staphylococcus aureus |     |     |     |
| Enterococcus faecalis |     |     |     |
| Escherichia coli      |     |     |     |
| Pseudomonas aerogenosa|     |     |     |
| Klebsiella pneumoniae |     |     |     |
| Salmonella typhi      |     |     |     |
| Shigella spp          |     |     |     |

Each value is expressed in mean±SD (n=6 in each test group). Co-aggregation is expressed in terms of Percentage (%) at 4, 18 and 24 h.

Neutralized supernatant of (pH 6.5) of all three Lactobacillus strains did not have any inhibitory activity effects. Among three Lactobacillus isolates CH3 isolate was bacteriocin and CH4, CH6 isolates were responsible for organic acid production. All the selected Lactobacillus isolates were subjected for the production of antimicrobial substances, CH4 and CH6 isolates were responsible for the production of only Bacteriocin, whereas CH3 isolate shown the production for both Bacteriocin and Organic acid against test pathogens.

Determination of minimal inhibitory concentration

All three Lactobacillus isolates were used for MIC test, the results clear that MIC for CH3 isolate was 50 µl against E. faecalis, S. typhi, S. aureus, k. pneumoniae, E. coli and p. aerogenosa, 100 µl for Shigella spp, for CH4 isolate 75 µl for k. pneumoniae, E. coli, p. aerogenosa and 100 µl for E. faecalis, S. typhi, S. aureus and Shigella spp and for CH6 isolate 128 µl for S. typhi, S. aureus, Shigella spp, E. faecalis and 100 µl for k. pneumoniae, E. coli, p. aerogenosa.

Auto and co-aggregation of probiotic Lactobacillus spp

The autoaggregation study was investigated for all three Lactobacillus isolates and different test pathogens based on their deposition capacity. The results exhibited that, among the three, CH3 isolate promptly noticed the highest percentage of auto aggregation after 24 h of the incubation period (51%) as compared to CH4 and CH6 isolates as shown in the table 6a.

The coaggregation results of the Lactobacillus isolates tested with different test pathogens as shown in the table 6b. This study is strain-specific as compared to aggregation, among the isolates, CH3, CH4 and CH6 isolates, showed the effective coaggregation with Shigella spp as 19.3, 17.4 and 20.4% respectively, similarly CH4 isolate showed the less coaggregation abilities with S. aureus, also the other test pathogens used.
Time-kill assays with cell-free culture supernatant (CFCs) of Lactobacillus spp on test pathogens

Time-kill assay showed the reduction in the cell counts of the different test pathogens in the presence of CFCs of each of Lactobacillus isolated; CH3, CH4 and CH6 covering 2-3 fractions of different incubation periods (6, 12, 18 and 24 h). The inhibition activity was more noticeable in the case of CH3 isolate as increasing in the incubation periods and as compared with other Lactobacillus CH4 and CH6 isolates. The study concluded that inhibitory substances like bacteriocin and organic acid presented in the CFCs of isolates were the responsible.

Cell surface hydrophobicity

Cell surface hydrophobicity was determined to study possible alliance between physicochemical property and its effective property on test pathogens used, in the selected Lactobacillus isolates, CH3 isolate (53%) was highest hydrophobic in nature, as compared to the other selected isolates were lesser or no hydrophobicity towards xylene from the control taken as 0%. Among the test pathogens used, Shigella spp and E. coli (29 and 24.4% respectively) exhibited better hydrophobicity percentage, Pseudomonas aerogenosa and Salmonella typhi (18.2 and 16.4% respectively), but Klebsiella pneumonia, Staphylococcus aureus and Enterococcus faecalis (8.4, 6.2 and 3% respectively) appeared less percentage of hydrophobicity as shown in table 7.

Quantification of organic acid and determination of pH value

The identified Lactobacillus species from Cheese samples CH3, CH4 and CH6 coagulated the skim milk and produced organic acids in the sterilized skim milk which were observed by the titrimetric method of different incubation periods. The results were showed in table 8.

DISCUSSION

The present study was aimed to isolate, identify and characterize the effective probiotic Lactobacillus spp from different companies of cheese samples, which are commercially available at the local milk vendors in city market of Gulbarga. The study exhibited that CH3, CH4 and CH6 Lactobacillus isolates (among 14 Lactobacillus isolates) considered as potential and novel probiotic bacteria to determine their...
antagonistic activity against common human test pathogens. On the basis of cultural and morphological characteristics [26] of all three selected Lactobacillus isolates which are separately isolated from different cheese samples. After gram staining the isolated bacteria were rod-shaped, convex, smooth, rough, non-motile and gram positive, (table 2) which cleared the member of Lactobacillus spp [27].

Optimum growth of the isolates was noted at pH 5.0 to 6.5 on MRS plates in anaerobic conditions, all the Lactobacillus isolates were catalase negative and oxidase negative, the results are in line with similar characterization criteria with Elizete and Carlos [28]. Most of the Lactobacillus isolates examined in this study (80-85%) were capable of fermenting glucose, sucrose, and sorbitol illustrates that they were able to grow in a variety of habitats using a different type of carbohydrates. Concerning to the better and potential probiotic bacteria must be capable of growing in acidic environments. The present work established the impelled gastric juice caused no appropriate decreases in viabilities of the Lactobacillus isolates; these isolates were likely to survive in an acidic environment of the intestine.

The pH in the human stomach ranges from 1.5 to 4.5 depending on the intervals of feeding, the types of food consumed, and the duration of food digestion, which can take up to 3-4 h. As the results in fig. 3 exhibits, all the collected Lactobacillus spp showed high and maximum isolates were tolerated to the range of pH and grow well in the acidic pH. These important observations are in agreement with those reported by Burns et al. [29]. Bile salts also consider an important factor for considering the Lactobacillus spp viability [30]. Isolates isolated from cheese samples was resistant to 0.5% bile salt, and all the isolates were able to survive and grow in 0.5% bile salt concentration up to the tested incubation period; 18h (fig. 4). This study also clears that, all of the isolates were also tolerate 1-6% NaCl and good growth at 1-5% of NaCl (fig. 5). In addition, inhibition of pathogenic strain growth is one of the adorable properties of probiotic bacteria. Pathogens can be combative through the production of antimicrobial compounds such as bacteriocin, organic acid, hydrogen peroxide, which competes for pathogen binding and receptor sites as well as for available nutrients and growth factors [17, 31]. Under our experimental circumstances, almost Lactobacillus isolates exhibited the clear zone for the pathogens used and presented different antagonistic activities. Over these results, we can expect human healthcare to benefit, improving protection against occurrences of diarrhea, enteric infection and improvement of our intestinal flora. As shown in the fig. 6, maximum Lactobacillus isolates showed sensitive to the antibiotic used. Hence, these Lactobacillus isolates were considered for further in vitro activities. Aggregation and co-aggregation study of the Lactobacillus isolates and different pathogenic strains having the role in several factors. The results of this study exhibited that, aggregation time increases as a concern and were highest at the 4h of incubation time period (table 6a and 6b).

CONCLUSION
Lactobacillus strains isolated in this study from the different dairy samples have in vitro properties that make them potential candidates for probiotic applications. Among the strains, Lactobacillus isolates from cheese predominantly exhibited interesting probiotic properties such as excellent pH and bile tolerance, aggregations, suppression of pathogen growth under in vitro conditions. Moreover, all tested strains were susceptible to a number of clinically effective antibiotics. These results collectively suggest that isolates from cheese have promising properties that are important for potential probiotics. Hence, more research is needed to exploit other potential probiotic properties of these strains. Further, in vivo trials are needed to determine whether they function as probiotics in real life situations for human health benefits.

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CONFLICT OF INTERESTS
We declare that no conflict of interest

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