

Original Research Article

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Characterization, Antimicrobial Activity and Antibiotic Susceptibility of Lactic Acid Bacteria Isolated from Food Samples

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ABSTRACT

Keywords

Isolate, Biochemical characterization, Antibiotic susceptibility, Antimicrobial activity, lactic acid bacteria.

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The present study was aimed at isolating *Lactobacilli* from food samples obtained from local areas of Allahabad. The isolates were biochemically characterized, subjected to Bergy's Manual of Determinative Bacteriology and PIBW in software. They were also tested for antimicrobial activity as well as antibiotic susceptibility. A total of 61 isolates were isolated and identified from 110 food samples. Out of 61 isolates 53.03% were characterized as *L. acidophilus*, 8.2% as *L. fermentum*, 21.31% as *L. plantarum*, 8.2% as *L. casei* and 3.2% were tentatively identified as *L. rhamnosus*. These results indicate that *L. acidophilus* is predominant in food samples collected from this sector followed by *L. plantarum*. All the isolates showed moderate to strong inhibition against *S. aureus* and *Aspergillus* strains. Maximum zone of inhibition (28 mm) was developed by about 59 % of isolates against *Aspergillus* strain. No inhibition was observed against *E. coli* strains by any isolate. Antibiotic susceptibility was measured against 2.5mg/ml, 5.0mg/ml and 10mg/ml concentrations of Ampicillin and Streptomycin. About 47.4% of isolates were resistant to lower concentrations of Ampicillin (5mg/ml) and Streptomycin (2.5mg/ml) but became sensitive as the concentration was increased to 10mg/ml of both antibiotics. About 44.2% of isolates were found to be highly sensitive against 10mg/ml Ampicillin and Streptomycin.

Introduction

Lactic Acid Bacteria is the most important group of acid producing bacteria in food industry. The industrial importance of the LAB is proved by their generally recognized as safe (GRAS) status, because of their contribution to the healthy microflora found in human mucosal surfaces (Patil *et al.*, 2010). They generally contains the genera in the order *Lactobacillales*, which includes *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Streptococcus*

as well as addition to *Carnobacterium*, *Oenococcus*, *Tetragenococcus*, *Enterococcus*, *Vagococcus*, and *Weissella* (Stiles and Holzappel, 1997). The genus *Lactobacillus* is very important among the various groups of lactic acid bacteria used in food fermentation and thus have a great economical importance (Schillinger, 1999). They are important part of our intestinal microflora, and their benefits for general state of human health is under serious

investigation (Salminen *et al.*, 1996). Various species of Lactobacilli are gaining importance in fermentation section of food industry because of their biotechnologically interesting properties (Roy *et al.*, 2000). They are also used as health-promoting probiotic ingredients since they have several therapeutic functions (Obergh *et al.*, 1998) including bile tolerance (Walker and Gilliland, 1983) antibiotic resistance (Curragh and Collins, 1992) and gastric juice tolerance (Kilara, 1982). *Lactobacilli* consist of a large and diverse group of Gram positive, rod shaped, catalase negative and nonspore forming bacteria which produces lactic acid on fermentation of carbohydrates as the main end product (Pelinescu *et al.*, 2009). The present study has been done to identify and characterize different strains of *lactobacillus spp.* isolated from different dairy and non dairy food samples.

Material and methods

Samples collection

A total of 110 samples including 10 samples each of raw milk, buttermilk, curd, cheese, prebiotics infant formulas, cabbage, cucumber, pumpkin, tomato, carrot, banana, apple were collected from different areas of Allahabad region. The dairy samples collected were both branded and non-branded in sterile bottles and kept cool until they could be taken to the laboratory. The vegetables and fruit samples were collected from local market. Reference culture of *Lactobacillus acidophilus* (MTCC 10307) and pathogenic strains (*Staphylococcus aureus*, *Salmonella enterica*, *Shigella flexneri*, *E.coli*, *Aspergillus*) for antimicrobial activity was obtained from MTCC, IMTECH, Chandigarh. The bacteriological media and antibiotics were obtained from Hi Media Laboratories Pvt. Ltd., India.

Isolation of Lactic acid bacteria

The dairy samples were aseptically weighed, homogenized and serially diluted in buffered peptone water. Diluted samples were pour-plated onto Mann Ragosa Sharpe agars (MRS) used for isolating LAB (Badis, *et al.*, 2004; Guessas and Kihal, 2004). The samples of fruits and vegetables were fermented in saline solution for 2 days at room temperature.. Then, 1 mL of sample was enriched in 9 mL MRS broth for 1-2 days (Naeem *et al.*, 2012). Subsequently serial dilutions were made using buffered peptone water. To prevent the growth of yeasts, the media were also supplemented with 100 mgL⁻¹ of cycloheximide before being incubated at the appropriate temperatures at 37°C for 24-48h. (Beukes *et al.*, 2001; Kalavrouzioti, *et al.*, 2005). Colonies were randomly selected and streak plating was further done to purify the strains (fig 1) which were subsequently kept in two different conditions including at 4°C for MRS plates and at -20°C for MRS broths supplemented by 20% glycerol for further use (Mathara *et al.*, 2004).

Identification of Lactic acid bacteria

Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics (Mohan kumar and Murugalatha, 2011; Chakraborty and Bhowal, 2015). The morphological characteristics of the isolates such as gram reaction, colour and type of colony, elevation and opacity were studied. The isolates were characterized on the basis of motility test, nitrate reduction test, catalase test, Methyl reduction and Voges- Proskauer test, citrate utilization, indole production in tryptone broth and arginine hydrolysis test as described in Bergy's Manual of systematic Bacteriology (Holt *et al.*, 1994). The identified lactobacillus genus was

further classified to species level based on their carbohydrate fermentation pattern (Singh and Sharma, 2009).

Physiological characterization of isolates

Optimum pH for growth of isolated colonies was determined by incubating 1 % (v/v) culture into MRS broth at varying pH ranging from 1-9. For the optimum temperature determination, the cultures were incubated at different temperature (10 °C, 37 °C, 42 °C) under anaerobic conditions. After 24 hr of incubation development of growth was measured by optical density at 560 nm against a control broth (Chakraborty and Bhowal, 2015). Effect of NaCl concentration on growth of isolates was also studied, fresh culture (71% v/v) of the isolate was inoculated into MRS broth with a varying NaCl concentration of 2-10%. After an incubation period of 24 hr the growth was determined by observing the turbidity (Abdulla *et al.*, 2015).

Sugar fermentation pattern

Different sugars were used for determining the fermentation pattern of lactobacillus genus. Sugars used were Maltose, Arabinose, Fructose, Mannitol, Sorbitol, Sucrose, Lactose. A sugar fermentation broth containing 1% of respective sugar and inverted Durham's tube was inoculated with isolated strain and incubated at 37°C for 24 hrs. The color change and gas production were recorded as positive or negative result. The cultures were further identified on the basis of sugar utilization pattern (Holt *et al.* 2004; Bhardwaj *et al.*, 2012).

Antimicrobial activity

Antimicrobial activity of isolated lactobacillus strains were studied against *Staphylococcus aureus*, *Salmonella enterica*,

Shigella flexneri, *E.coli* and *Aspergillus* using agar well diffusion method (Topisirovic *et al.*, 2006). 500mL Erlenmeyer's flasks each containing 200 ml MRS broth was autoclaved at 121°C for 15 minutes and inoculated with colony of a LAB isolate grown on MRS agar. The inoculated flasks were incubated at 37°C for 2-3 days under stationary condition. Then centrifuged at 10000 rpm for 10 min. After incubation, 2 ml of each fermented culture broth and supernatant was taken to test the antimicrobial activity. The plates were incubated at 37°C for 24 hr and inhibition zones were measured.

Antibiotic susceptibility test

Disk diffusion method was followed to determine the sensitivity of the isolated culture to antibiotics such as Ampicillin and Streptomycin at varying concentrations (2.5-10mg/ml). The test inoculum was prepared by incubating the isolated culture into MRS broth at 37°C for 12 hr and 100 µl of it was inoculated to Nutrient agar plates by spread plate method. Four wells were made in each of the plates and were filled with 100 µl of selected antibiotics each of different concentrations. The plates were then incubated for 24 hr at 37°C and the zone of inhibition was measured. (Chakraborty and Bhowal, 2015).

Results and Discussion

Isolation and identification of *Lactobacillus* species

61 colonies were isolated by observing colony morphology, pure cultured and stored in soft agar tube (Table 1). All the colonies were selected on the basis of colony morphology and gram staining. The strains were showing different colonial morphology i.e. colour, size, margin, shape,

gram reaction and opacity. Isolates obtained from curd samples were found to be gram positive, small in size, creamy white and shiny in appearance with raised elevation and translucent colony whereas isolates from raw milk samples were also gram positive (fig 2) small translucent but were white shiny in appearance with convex elevation. About 26% of the isolates showed large size colonies with creamy white and non-shiny colour and opaque appearance. (Chakarborty and Bhowal, 2015) have also reported that isolated colonies of *Lactobacilli* showed creamy white colour, circular shape, low convex with entire margins appearance.

Isolated strains of lactobacillus species have shown a negative pattern for catalase activity, nitrate reduction, indole test, motility, arginine hydrolysis and acid from glucose. These are the essential characteristics of lactobacillus group. However different strains showed variable results in terms of citrate utilization, methyl red and Voges-Proskauer test. The results are in accordance with Pundir *et al.*, (2013), Bhardwaj *et al.*, (2012), (Chakarborty and Bhowal, 2015).

Physiological Characterization

Growth at different temperature and pH

The growth of organism was tested against influence of pH and found to be the highest at pH 5-7 and a little growth at pH 8 and pH 3. Pundir *et al.*, (2013) have also reported that LAB isolates were able to grow in pH range 4-7. (Chakarborty and Bhowal, 2015) have also reported that optimum pH value for Lactic acid bacteria was between 5.5 to 6.5. Hence it can be concluded that

lactobacilli can survive in extreme acidic as well as alkaline environment.

Similarly for growth at different temperature all the isolates showed luxuriant growth and turbidity at 37° C but little and no growth at 42°C and 10°C respectively (Table 3).

Growth at different NaCl concentration

The effect of different NaCl concentrations (2, 4, 6, 8 and 10%) was observed in LAB in term of turbidity (Table 3). The 4% NaCl concentration was found to be optimum for LAB growth. As the concentration was increased from 6-10%, no turbidity was observed. Pundir *et al.*, (2013) have also reported that Lactic acid bacteria isolates were able to tolerate 1-6.5% NaCl concentration.

Sugar Fermentation Pattern

Different isolates showed different types of sugar utilization patterns (Table 5). On comparing the sugar utilization patterns with those given for *Lactobacillus* species in the Bergey's Manual of Determinative Bacteriology, the isolates were tentatively identified as *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. casei* and *L. rhamnosus*. The data obtained for genus and species identification comprising a number of morphological, physiological, biochemical and sugar utilization pattern tests were also subjected to software called PIBWin. (Bryant, 2004). Out of 61 isolates 53.03% were characterized as *L. acidophilus*, 8.2% as *L. fermentum*, 21.31% as *L. plantarum*, 8.2% as *L. casei* and 3.2% were tentatively identified as *L. rhamnosus* (Table 5). Similar use of software for identification of species has been reported by Bhardwaj *et al* (2012).

Table.1 Isolation of bacteria from food samples

| Sources | No. of samples | No. of Isolates | Codes of Isolates | Growth on MRS agar |
|-----------------------------------|----------------|-----------------|--|--------------------|
| Curd | 10 | 9 | C1, C2, C4, C5, C6, C7, C8, C9, C10 | +++ |
| Raw Milk | 10 | 9 | RM1, RM2, RM3, RM4, RM6, RM7, RM8, RM9, RM10 | +++ |
| Cheese | 10 | 5 | CH3, CH4, CH5, CH6, CH7 | ++ |
| Probiotics | 10 | 3 | Y, Z, F | + |
| Buttermilk | 10 | 7 | BM1, BM2, BM3, BM4, BM5, BM6, BM7 | +++ |
| Apple | 10 | - | | - |
| Banana | 10 | - | | - |
| Cabbage | 10 | 5 | CB1, CB4, CB5, CB6, CB7 | ++ |
| Cucumber | 10 | 8 | Cu1, Cu2, Cu3, Cu4, Cu5, Cu6, Cu7, Cu8 | +++ |
| Carrot | 10 | 7 | Ca3, Ca4, Ca5, Ca6, Ca8, Ca9, Ca10 | +++ |
| Pumpkin | 10 | 8 | P2, P3, P4, P5, P6, P7, P8, P9 | +++ |
| <i>L.acidophilus</i> (MTCC 10307) | | | | +++ |

+=Minimum growth; ++=Moderate growth; +++=High growth; -=No growth

Table.2 Morphological characteristics of cultures isolated on MRS agar

| Isolates | Gram Reaction | Type of colony | Color | Margin | Elevation | Opacity |
|--|---------------|----------------|-------------------------|--------|-----------|-------------|
| C1,C2,C4,C6 | Gram + | Small | Creamy white, shiny | Entire | Raised | Translucent |
| RM1, RM2, RM3, RM4, RM6, RM7, RM8, RM9, RM10 | Gram + | Small | White, shiny | Entire | Convex | Translucent |
| C5, C7, C8, C9, C10 | Gram + | Small | Off-white | Entire | Raised | Translucent |
| CB1, CB4, CB5, CB6 | Gram + | Large | Off-white, non-shiny | Entire | Flat | Opaque |
| Y, Z, F | Gram + | Small | Off-white, non-shiny | Entire | Flat | Opaque |
| BM1, BM2, BM3, BM4, BM5, BM6, BM7, P2 | Gram + | Small | Creamy-white, non-shiny | Entire | Convex | Opaque |
| Cu1, Cu2, Cu3, Cu4, Cu5, Cu6, Cu7, Cu8, Ca5, Ca6, Ca8, Ca9, Ca10, P3, P4, P5, P6, P7, P8, P9, CB7, CH3, CH4, CH5, CH6, CH7 | Gram + | Large | Creamy-white, non-shiny | Entire | Flat | Opaque |
| <i>L.acidophilus</i> (MTCC 10307) | Gram + | Large | Creamy-white, non-shiny | Entire | Flat | Opaque |

Table.3 Physiological characterization of isolates

| Isolates | Effect of temperature | | | Effect of pH | | | | | Effect of NaCl% | | | | |
|---|-----------------------|------|------|--------------|---|----|---|---|-----------------|----|---|---|----|
| | 10°C | 37°C | 42°C | 1 | 4 | 5 | 8 | 9 | 2 | 4 | 6 | 8 | 10 |
| C1,C2,C4,C7,C8,C9,C10,RM4,RM6,RM7,RM8,BM1,BM2,BM3,BM4,BM5,Y,Z,F,Ch3,Ch4,Ch5,Ch6,Ch7, Ca3,Ca4,Ca5,Ca6,Ca7,Ca8,Ca9,Ca10,Cu1,Cu2,Cu3,Cu6,Cb1,Cb5,Cb6,P3,P4,P5,P7 | + | ++ | + | - | + | ++ | - | - | + | ++ | + | - | - |
| C6,RM1,RM2,RM3,BM6,BM7,Cb4,Cb7,P6,P8,P9,Cu4,Cu5 | - | ++ | + | - | + | ++ | + | - | + | ++ | - | - | - |
| C5,Cu7,Cu8,RM9,RM10 | - | ++ | + | - | - | ++ | - | - | + | ++ | - | - | - |
| <i>L.acidophilus</i> (MTCC 10307) | + | ++ | - | - | + | ++ | - | - | + | ++ | - | - | - |

+=able to grow; += maximum growth; - = no growth

Table.4 Biochemical characteristics of isolates

| Isolates | Catalase | Nitrate reduction | Citrate utilization | Methyl Red | Voge's Proskauer | Indole Test | Arginine Hydrolysis | Motility | Acid from Glucose |
|---|----------|-------------------|---------------------|------------|------------------|-------------|---------------------|----------|-------------------|
| C1,C2,C4,C5,C8,RM6,RM7,RM8,RM9,RM10,CH3,CH4,CH5,CH6,CH7,BM1,BM4,BM5,BM6,BM7,CB1,CB4,CB5,CB6,CB7,Cu1,Cu3,Cu4,Cu5,Cu6,Cu7,Cu8,Ca4,Ca9,Ca10,P5 | - | - | + | - | + | - | + | - | + |
| C6,C7,C9,BM2,BM3, | - | - | - | - | + | - | + | - | + |
| C10,RM3,RM4,Y,Z,F,Cu2,Ca5,Ca7,P3,P7,P8,P9 | - | - | + | - | - | - | + | - | + |
| Ca3,Ca6,Ca8,P4,P6 | - | - | - | + | + | - | + | - | + |
| RM1,RM2 | - | - | - | - | - | - | + | - | + |
| <i>L.acidophilus</i> (MTCC 10307) | - | - | + | - | + | - | + | - | + |

+= Positive reaction; -= Negative reaction or no reaction

Table.5 Sugar fermentation pattern by isolates

| Isolates | Arabinose | Lactose | Maltose | Mannitol | Sucrose | Sorbitol | Ribose | Probable Identified Organism |
|--|-----------|---------|---------|----------|---------|----------|--------|-------------------------------|
| C1,C2,C4,C5,C8, RM6, RM7, RM8, RM9, RM10, CH3, CH4, CH5, CH6, CH7, BM1, BM4, BM5, BM6, BM7, CB1, CB4, CB5, CB6, CB7, Cu1, Cu3, Cu4, Cu5, Cu6, Cu7, Cu8, Ca4, Ca9, Ca10, P5 | - | + | - | + | + | - | - | <i>L.acidophilus</i> (59.03%) |
| C6,C7,C9, BM2, BM3 | V | + | + | + | - | - | + | <i>L.fermentum</i> (8.2%) |
| C10, RM3, RM4, Y, Z, F, Cu2, Ca5, Ca7, P3, P7, P8, P9 | V | + | + | + | + | + | + | <i>L.plantarum</i> (21.31%) |
| Ca3, Ca6, Ca8, P4, P6 | - | + | + | + | + | + | - | <i>L.casei</i> (8.2%) |
| RM1, RM2 | - | + | + | + | - | + | + | <i>L.rhamnosus</i> (3.2%) |

V = variable fermentation; + = fermentation; - = no fermentation

Table.6 Antimicrobial activity of isolates

| Groups | Isolates | Diameter of zone of inhibition zone (DIZ mm) | | | | | |
|--------|--|--|-----------------|-------------------|-----------------|---------------|--------------------|
| | | % of isolates | <i>S.aureus</i> | <i>Salmonella</i> | <i>Shigella</i> | <i>E.coli</i> | <i>Aspergillus</i> |
| I | C1,C2,C4,C5,C8, RM6, RM7, RM8, RM9, RM10, CH3, CH4, CH5, CH6, CH7, BM1, BM4, BM5, BM6, BM7, CB1, CB4, CB5, CB6, CB7, Cu1, Cu3, Cu4, Cu5, Cu6, Cu7, Cu8, Ca4, Ca9, Ca10, P5 | 59% | SI 15 | MI 4 | MI 6 | NI | VSI 28 |
| II | C6,C7,C9, BM2, BM3, C10, RM3, RM4, Y, Z, F, Cu2, Ca5, Ca7, P3, P7, P8, P9 | 29.5% | SI 18 | MI 2 | MI 5 | NI | SI 15 |
| III | Ca3, Ca6, Ca8, P4, P6 | 8.19% | SI 5 | NI | NI | NI | SI 10 |
| IV | RM1, RM2 | 3.27% | MI 4 | NI | NI | NI | MI 5 |
| V | <i>L. acidophilus</i> (MTCC 10307) | - | 22 | 8 | 11 | 5 | 28 |

VSI = very strong inhibition; SI = strong inhibition; MI = moderate inhibition;

NI = no inhibition

Table.7 Antibiotic susceptibility of isolates

| Groups | Antibiotics Isolates | % of isolates | Diameter of zone of inhibition zone (DIZ mm) | | | | | |
|--------|--|---------------|--|-----|----|----------------------------|-----|----|
| | | | Ampicillin (in mg/ml) | | | Streptomycin (in mg/ml) | | |
| | | | 2.5 | 5.0 | 10 | 2.5 | 5.0 | 10 |
| I | C1,C2,C4,C5,C8, RM6, RM7, RM8, RM9, RM10, CH3, CH4, CH5, CH6, CH7, BM1, BM4, BM5, BM6, BM7, CB1, CB4, CB5, CB6, CB7, Cu1, Cu3, | 44.2% | 5 | 10 | 12 | 4 | 8 | 15 |
| II | Cu4, Cu5, Cu6, Cu7, Cu8, Ca4, Ca9, Ca10, P5, C6, C7, C9, BM2, BM3, C10, RM3, RM4 Y, Z, F, Cu2, Ca5 | 36% | NI | NI | 5 | NI | 5 | 10 |
| III | Ca3, Ca6, Ca8, P4, P6, RM1, RM2 | 11.4% | NI | NI | 5 | NI | 10 | 15 |
| IV | <i>L.acidophilus</i> (MTCC 10307) | - | 6 | 12 | 16 | 8 | 12 | 19 |

NI = no inhibition

Fig.1 Isolation of *Lactobacillus* spp. from food samples

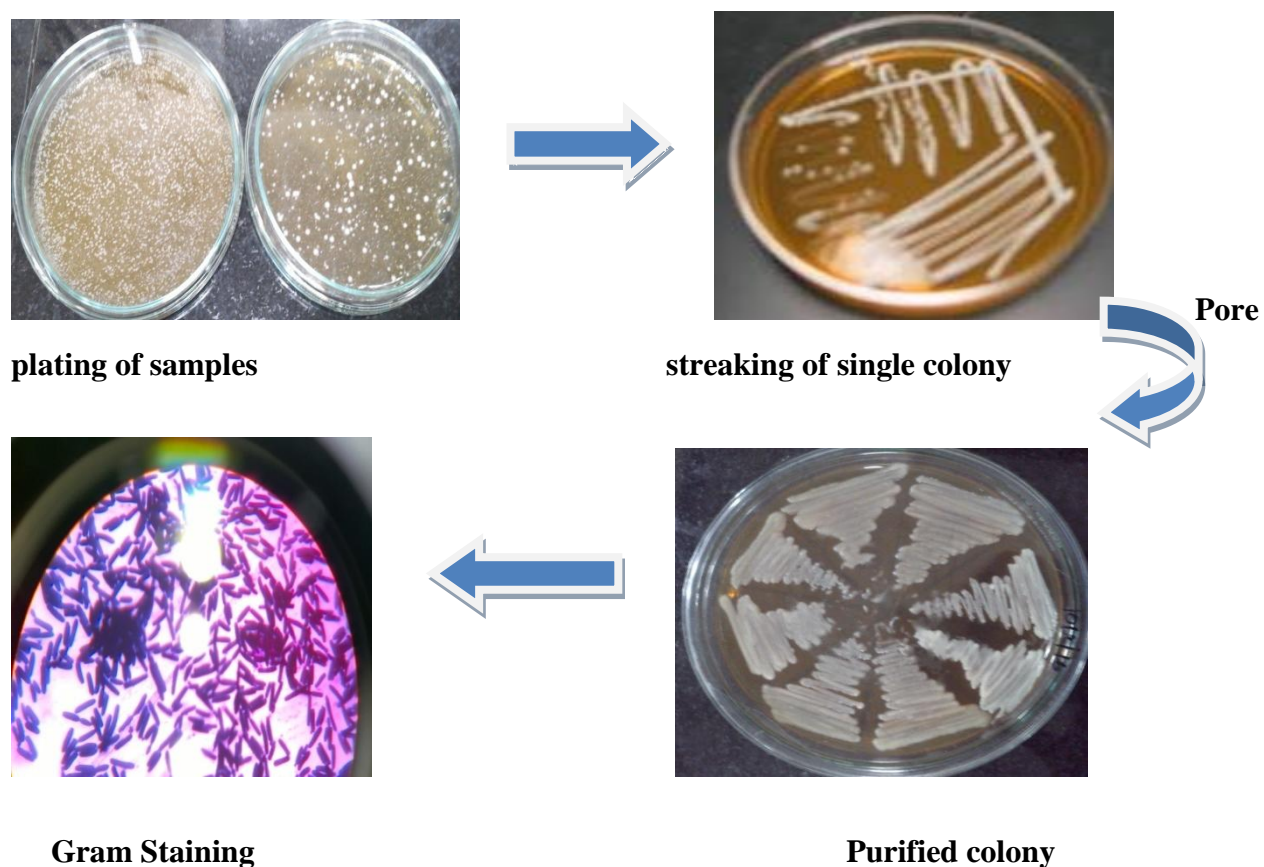


Fig.2 Gram Staining: (a) Buttermilk, (b) Raw Milk, (c) Pumpkin

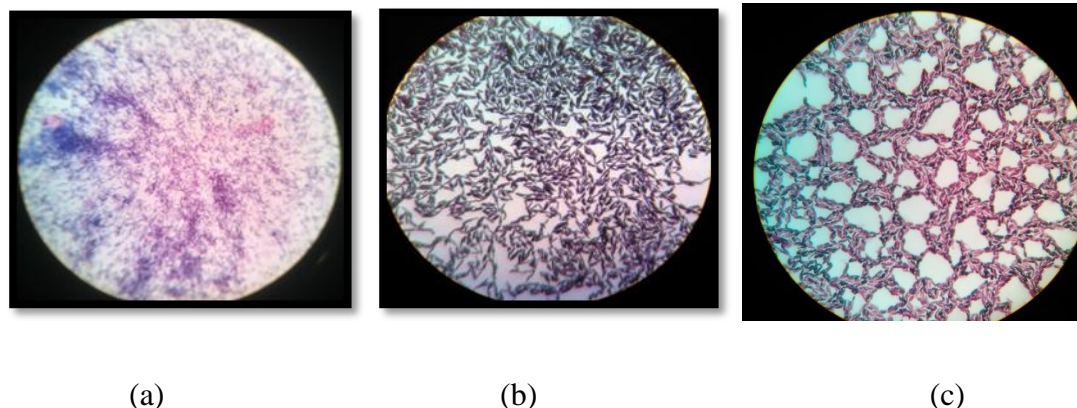
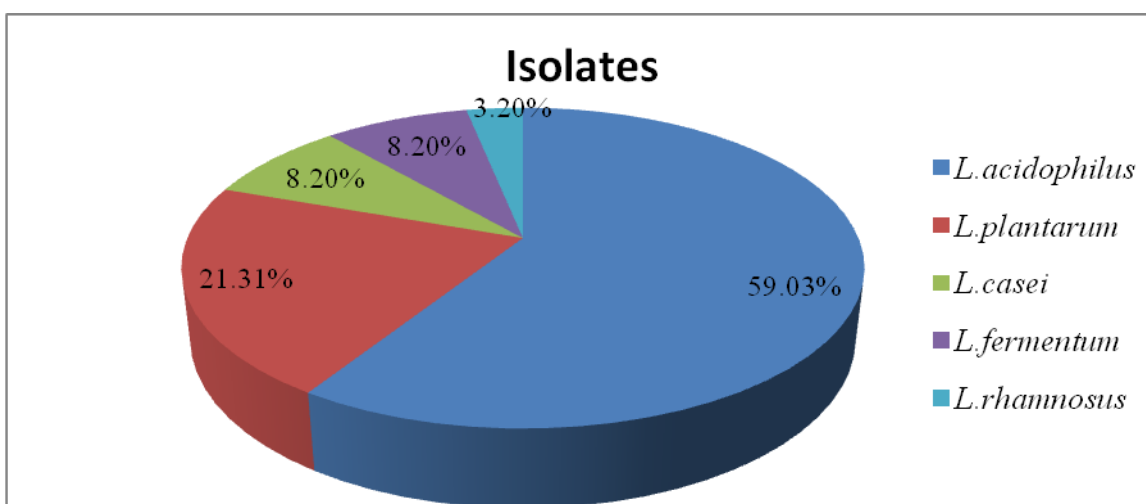


Fig.3 Prevalence of *Lactobacillus* strains



Antimicrobial Activity of isolates

Table 6 represents the antimicrobial effect of the isolated strains against different pathogenic microorganism. The antimicrobial activities exhibited by *Lactobacillus* species indicates that the cell free solution of isolated *Lactobacillus* species were able to inhibit the growth of all the test microorganisms except *E. coli*. Maximum inhibition zone (28 mm) was observed against *Aspergillus* strain followed by *S.aureus* (18mm). Pundir *et al.*, (2013) have also reported that *Lactobacillus* showed maximum zone of inhibition (31mm) against *Aspergillus* whereas

minimum zone of inhibition was observed against *E.coli* and *Salmonella*.

Antibiotic susceptibility of isolates

The antibiotic susceptibility of the isolates against different concentrations of Ampicillin and Streptomycin is given in Table 7. About 44.2% of isolates were found to be highly sensitive against 10mg/ml Ampicillin and Streptomycin but about 47.4% of isolates were resistant to lower concentrations of Ampicillin (5mg/ml) and Streptomycin (2.5mg/ml). Zhou *et al.* (2005) has also reported high resistance of *Lactobacilli* to streptomycin. The findings

are in accordance with previously reported results of Belletti *et al.*, 2009, where most of the tested *L.acidophilus* and *L.plantarum* strains were susceptible to Ampicillin and Streptomycin.

In conclusion, on the present study, a total of 61 isolates were obtained from dairy and vegetables sample. The isolates were identified as *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L.casei* and *L. rhamnosus*. *L. acidophilus* was the most prevalent species in the samples studied. The strains were found to have antimicrobial activity against pathogens except *E. coli*. The isolates were resistant to lower concentrations of antibiotics studied but as the concentration was increased the isolated strains become susceptible. These characteristics show that the isolates have potential to be used as a natural preservative and for probiotic applications.

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