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STUDIES OF PROBIOTIC EFFECTS OF INDIAN FERMENTED FOOD AND DAIRY PRODUCTS

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ABSTRACT

Lactic acid bacteria (LAB) are a group of Gram-positive, anaerobic, aerotolerant bacteria. They are benevolent residents of the normal human gut flora exhibiting a symbiotic relationship. LABs breakdown lactose into lactic acid and other organic acids, thereby, contributing to enhanced food digestibility. Current work was undertaken with an objective to evaluate the probiotic (health restoring) effect of Indian fermented food and dairy products and understanding the association of longevity with fermented foods. Isolation of LABs from idli batter, ambil and sheep milk was undertaken. A total of six isolates were isolated from the samples, which were then subjected to microscopic and biochemical characterization tests. All the isolates were found to belong to the genus Lactobacillus. Furthermore these isolates were examined for their probiotic properties like tolerance against pH, NaCl and antimicrobial activity. The isolates showed promising probiotic effects when compared with commercial Lactobacillus strain. Thus numerous probiotic products can be developed from these isolates as well they can furthermore be evaluated for the anti-cancer activity.

***Keywords*: Ambil, Fermented foods, Idli, Lactic acid bacteria, Probiotic, etc.**

I. INTRODUCTION

Lactic acid bacteria (LAB) are widely used in many fields of human activity. They have been efficiently used for thousands of years to produce fermented foods with improved preservation properties as well as with characteristic flavours and textures different from the original food. LABs have proven to be far more advantageous than just for the food preservation and dairy products [1].

LABs are extensively used around the globe in a range of industrial and biotechnological applications including;

- Brewing industry – LABs triggers malolactic fermentation (MFL) thereby yielding a characteristic flavour and aroma to brewed products like wine [2]
- Food industry –employed as a bioprotectant in meat industry [3,4]
- Dairy industry – preservation and enhancement of flavour of dairy products such as fermented milks (yoghurt, cultured buttermilk) and cheese [1]
- Antimicrobials – LABs are observed to secrete an array of antimicrobial compounds like nisin, bacteriocin, often employed as a preservative against plant pathogens [5]
- Probiotics - as a food medicine, probiotic drinks and functional foods [6]
- Biofertilizers – they improve soil quality, control disease and promote plant growth [7]

The new era of medicine leans towards preventive healthcare measures, here LABs provide promising probiotic activity. LABs belong to a group of beneficially acting bacteria (also termed as protective microflora) that

IJARSE
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colonize mainly in the gut mucosal membrane. Mucous membranes in the body are in direct contact with the outside environment. Through mucous membranes, the gut microflora is in permanent contact with different antigens. The structure, function and development of the whole immune system of the host mucous are markedly affected by this indigenous microflora. Protective microflora prevents pathogens from adhering by competition for substrates and places of adhesion. They simultaneously secrets antibacterial substances and stimulate the production of specific antibodies and mucous. The early colonization of the gut with living microorganisms is important for development of the gut protection barrier. LABs stimulate the production of secretory IgA, affect the targeted transportation of the luminal antigens to Peyer's patches and increase the production of IFN-γ. Thus LABs are responsible for eliminating the damage causing factors from the gut microenvironment by stimulating local and systemic immune responses and thereby assists in restoring the gut wall integrity [6].

A great research is been ongoing on the probiotic role of LABs. Researchers have attempted to isolate LABs from various dairy products (milk, curd, and cheese), non-dairy products (ripened fruits, cucumber and soybean, seafood, wine, fruit juices), faecal samples, soil etc. [8, 9]. Different species of LABs have adapted to grow under widely different environmental conditions as depicted in Table 1.

Table 1: Summary of varied sources of isolation and types of LABs

Sr. No.	Source of isolation	Types of LABs	References
1	Curd	*Lactobacillus*	[10]
2	Milk	*Lactobacillus*	[11]
3	Ripened mulberries	*Lactobacillus, Leuconostoc*	[12]
4	Curd and cucumber	*Lactobacillus, Weissella, Pediococcus*	[13]
5	Fermented soybean milk	*Lactobacillus*	[14]
6	Grape and must wine	*Leuconostoc, Oenococcus, Lactobacillus,*	[15]
8	Fish	*Streptococcus, Leuconostoc, Lactobacillus, Carnobacterium Lactococcus*	[16]
9	Plant surface	*Lactobacillus*	[17]
10	Gastrointestinal tracts of gnotobiotic animals	*Lactobacillus*	[18]
11	Honeycomb	*Lactobacillus*	[19]
12	Soil	*Lactobacillus, Lactococcus, Enterococcus, Leuconostoc and Weissella*	[20]

By reviewing the literature on LABs and its potential application as a nutraceutical, we streamlined the objective of our research work. We selected Indian traditional fermented foods *viz.* idli batter and ambil as a source for isolation of LABs. Also, the isolation of LABs from sheep's milk was relatively less studied. Hence, we also attempted to isolate the cultures from sheep's milk. The isolates were then subjected to morphological and biochemical characterization tests followed by evaluation of their probiotic potential.

IJARSE
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ISSN (P) 2319 - 8346

II. MATERIALS AND METHODS

2.1 Materials

All the chemicals used for the study like peptone, beef extract, yeast extract, glucose, sodium acetate trihydrate, polysorbate 80, dipotassium dihydrogen phosphate, triammonium citrate, magnesium sulfate, manganese sulfate, sodium citrate (dehydrated), ammonium dihydrogen phosphate, potassium hydroxide, urea and skim milk were procured from Himedia Pvt. Ltd., Mumbai.

2.2. Microbial Isolation

All the isolation work was carried out under strict aseptic and sterile conditions using the selective MRS media for *Lactobacilli*. Three samples chosen for the isolation purpose were fermented idli batter, traditional fermented Maharashtrian food, ambil and sheep's milk.

- **Isolation of LABs from fermented idli batter**

  Home-made idli batter was prepared by mixing rice flour and black lentils properly and allowing it to ferment overnight. The batter sample was then transferred to a sterile container. The sample was suitably diluted using sterile saline and aseptically transferred under Vertical Laminar Air Flow Unit (Imset) on sterile MRS agar by traditional spread plate microbial technique. The plates were then incubated in a Bacteriological Incubator (Kumar) for 48 h at 37 ºC.

- **Isolation of LABs from ambil**

  Ambil is a Maharashtrian fermented food prepared by ragi flour, water, salt and buttermilk. Ragi flour was mixed with hot water and salt was added. After thorough mixing, buttermilk was added to the mixture and ambil was prepared. The ambil sample was then transferred to a sterile container. The sample was suitably diluted using sterile saline and aseptically transferred under Vertical Laminar Air Flow Unit (Imset) on sterile MRS agar by traditional spread plate microbial technique. The plates were then incubated in a Bacteriological Incubator (Kumar) for 48 h at 37 ºC.

- **Isolation of LABs from sheep's milk**

  Sheep's milk was collected from a dairy and transferred to a sterile container. The sample was suitably diluted using sterile saline and aseptically transferred under Vertical Laminar Air Flow Unit (Imset) on sterile MRS agar by traditional spread plate microbial technique. The plates were then incubated in a Bacteriological Incubator (Kumar) for 48 h at 37 ºC.

2.3 Microbial Purification

The isolates so obtained on the MRS agar plates were then purified till a single homogeneous colony was obtained per plate by employing streak plate technique under strict sterile and aseptic conditions. Appropriate controls were also run parallel with the test samples under the same experimental conditions.

2.4 Maintenance and Preservation of Isolates

All the six isolates were revived by inoculating the isolates individually into separate MRS broth and incubated at 37 ºC for 80 rpm for 24 h. The cultures were then immediately streaked onto the surface of MRS agar slopes

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

and further incubated at 37 ºC for 24-36 h, after which they were stored for 15 days at 4 ºC. Occasional subculture of the isolates was done after every 15 days. Simultaneously the glycerol stocks of the isolates were prepared using 35% v/v glycerol and were stored in Deep Freezer -20 ºC (REMI).

2.5. Morphological Characterization of the Isolates

Each of the isolates was streaked onto the MRS agar plates and incubated at 37 ºC for 24-36 h and visually observed for their morphological features.

2.6. Microscopic Characterization of the Isolates

- **Gram's staining**

  Gram staining of the isolates was performed using crystal violet as a primary stain, Gram's iodine solution as a mordant, 70% acetone as decolorizer and safranin as a counter stain. Heat-fixed smear of all the isolates was made on microscopic slides. Few drops of crystal violet was added as a primary stain and iodine solution was added immediately. Iodine helps to trap the crystal violet in the cell membrane. The smear was then rinsed with distilled water and safranin was added as a counter stain. The slides were then rinsed with water and the isolates were then observed under a compound microscope (Olympus) using 100X oil immersion lens.

- **Endospore test**

  A heat fixed bacterial smear of all the isolates was prepared on their respective slides and a filter paper was placed on it. Malachite green stain was added on the filter paper and the slides were kept above the bunsen burner for 5-10 min. The slides were then removed and washed with water. Few drops of counter stain safranin were added on each slide and were allowed to react for 30 seconds. The slides were washed again and observed under a microscope (Olympus) using 100X oil immersion lens.

- **Motility test**

  A loopful of each of the culture was taken on a microscopic slide and observed under a microscope (Olympus) using 100X oil immersion lens for its motility.

2.7. Biochemical Characterization of the Isolates

The isolates were then subjected to a series of biochemical tests. The tests were performed in accordance with the Bergey's Manual **[**21**].**

- **Catalase test**

  A loopful culture of the isolated colonies was immersed into a drop of 30% hydrogen peroxide, placed on a clean glass slide. Results were interpreted by observing the presence or absence of bubbles.

- **Oxidase Disc test**

  5µl of the culture was placed on the separate oxidase discs and were observed for its color change.

- **Indole test**This test was used to determine the ability of organisms to convert tryptophan into indole. This test is performed by adding 5 drops of Kovac's Indole reagent in a 24-48 h old culture grown in peptone broth. The culture was then observed for its color change.

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

- **Methyl-red (MR) test**
- This test was used to determine whether microbes perform mixed acid fermentation when supplied with glucose. Microbes were grown in MR-VP broth for 24-48 h whose composition is as follows: peptone-7 g/l, glucose-5 g/l, dipotassium phosphate-5 g/l. The culture was then observed for color change by adding 5 drops of methyl-red solution per 1ml of culture broth.
- **Voges–Proskauer (VP) test**

  This test was used to determine acetoin production in the bacterial culture broth. The microbes were inoculated in MR-VP broth mentioned above. 0.6 ml of 5% α-naphthol was added followed by 0.2 ml of 40% potassium hydroxide per ml of 24-48 h old microbial culture. The cultures were then allowed to remain undisturbed for 10-15 minutes and its color was observed.
- **Citrate Utilization test**

  This test is used to determine the ability of microbes to utilize sodium citrate as it's sole carbon source. The microbial culture was streaked onto citrate agar medium slants and growth was observed after 24-48 h. The composition used for citrate agar medium is as follows-sodium chloride (5 g/l), sodium citrate-dehydrated (2 g/l), ammonium dihydrogen phosphate (1 g/l), dipotassium phosphate (1 g/l), magnesium sulfate (0.2 g/l), bromothymol blue (0.08 g/l), agar (15 g/l).
- **Sugar fermentation test**

  This test was used to determine different carbohydrate utilization of microbes. The culture was inoculated in nutrient broth containing phenol red as an indicat**o**r dye and 1% solution of glucose, fructose and lactose were separately added in culture medium and color change was observed after 24-48 h of incubation.
- **Urease test**

  This test is used to determine whether a microorganism can produce urease for ammonia production. The culture was grown in urease broth for 24-48 h and observed for media color change. After incubation period, media was observed for color change due to the change in its alkalinity. The urease broth composition is as follows- monopotassium phosphate (9 g/l), dipotassium phosphate (9.5 g/l), yeast extract (0.1 g/l), phenol red powder (0.01 g/l), urea (20 g/l).
- **Milk coagulation test**

  This test was performed by inoculating 1% of 24-48 hour old microbial culture in 10% skim milk and pH was recorded before and after an incubation period of 72 h.

2.8. Evaluation of Probiotic Activity

- **NaCl tolerance test**

All the seven isolates were grown in MRS medium containing varying concentrations of NaCl *viz*, 3%, 5%, 7% and 9% and were incubated in rotary shaker at 37 °C for 48 h. The growth was observed in all the isolates and optical density of the culture was measured at 660 nm [8].

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

- **pH tolerance test**

  All the seven isolates were grown in MRS medium of varying pH 3, 5, 7 and 9 and were incubated in rotary shaker at 37 °C for 48 h. The growth was observed in all the isolates and optical density of the culture was measured at 660 nm [8].

- **Antimicrobial activity**

This test was performed using agar well diffusion method. A 48 h old *E.coli* culture was spread onto the surface of nutrient agar plates by pour plate technique. Wells were bored using a borer and bacterial culture was poured in the wells. The plates were then kept for incubation at 37 °C for 48 h [22].

III. RESULTS AND DISCUSSION

3.1. Microbial Isolation and Purification

Samples were collected from idli batter, fermented ambil and Sheep's milk. All the three specimens were serially diluted and streaked on the MRS agar plates.

The colonies that were obtained from the first plating were further subcultured by taking a loopful of a colony and the growth was obtained as shown in Fig. 1. We obtained a total of six of the purified isolates from the three different samples employed. The isolates were named as enumerated below in Table 2.

Table 2: Number and nomenclature of the isolates with respect to their sample

Sr No	Sample	Number of isolates	Nomenclature
1	Idli batter	01	JNEC/ID/01
2	Sheep's milk	01	JNEC/SHE/01
3	Ambil	04	JNEC/AMB/01, JNEC/AMB/02, JNEC/AMB/03 and JNEC/AMB/04


JNEC/ID/01


JNEC/SHE/01

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

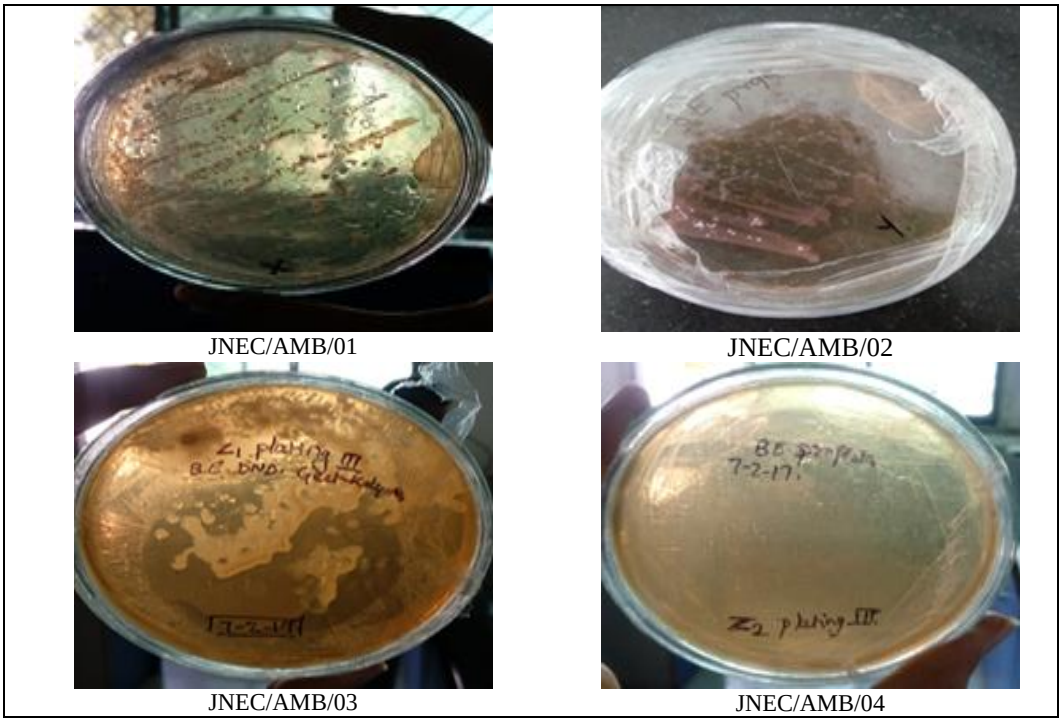

Figure 1: isolated microbial colonies onto MRS agar

3.2. Morphological Characterization of the Isolates

All the six isolates along with one standard *Lactobacilli* culture (Sporolac Tablet, UNI SANKYO) were evaluated morphologically to study its morphological traits. Table 3 describes comparative morphological traits of all the isolates.

Table 3: Morphological characterization of the isolates

Parameter	JNEC/ID/01	JNEC/SHE/01	JNEC/AMB/01	JNEC/AMB/02	JNEC/AMB/03	JNEC/AMB/04	LAB
Shape	Round	round	rod	Rod	whitish milky	fibrous	round
Margin	Spread	sharp	sharp	Sharp	spread	fibrous	sharp
Color	White	white	white	Brown	white	white	white
Transparency	opaque	opaque	opaque	Opaque	opaque	opaque	opaque

3.4. Microscopic Characterization

All the six isolates along with one standard *Lactobacilli* culture were evaluated microscopically. Table 4 describes comparative microscopic traits of all the isolates. It can be clearly observed from the Table 4, that all the isolates exhibit similar gram's nature except JNEC/AMB/01 and JNEC/AMB/02 as the standard *Lactobacillus* strain. Results of endospore staining and motility test indicate isolates displaying similar microscopic traits as compared to the *Lactobacillus* genus.

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

Table 4: Microscopic characterization of the isolates

Tests	**JNEC/ID/01**	**JNEC/SHE/01**	**JNEC/AMB/01**	**JNEC/AMB/02**	**JNEC/AMB/03**	**JNEC/AMB/04**	***Lactobacillus***
Gram staining	Gram positive	Gram positive	Gram negative	Gram negative	Gram positive	Gram positive	Gram positive
Endospore	Negative	negative	negative	negative	Negative	negative	negative
Motility	Negative	negative	negative	negative	Negative	negative	negative

3.5. Biochemical Characterization

- **Catalase Test**

This test demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The catalase test was performed on all the seven isolates and all the isolates gave negative results for this test. A negative catalase test indicates the cultures belong to the class of facultative microbes. Our results coincide with the results reported by Chauhan et al. (2016), Chakraborty et al. (2015), Borase et al. (2015) and Halder et al. (2015) [8,10,22,23].

- **Oxidase Test**

Oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. If the enzyme is present, it oxidizes the reagent to purple color end product. If there is no enzyme present, the reagent remains reduced and is colorless. The oxidase test was performed on all the seven isolates and all the isolates gave negative results for this test. Again this negative oxidase test indicates the facultative nature of isolates. Our results coincide with the results of Halder et al. (2015) [23].

- **Indole Test**

This test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. The Indole test was performed on all the seven isolates and all the isolates gave negative results for this test indicating inability of isolates to degrade tryptophan. Our results coincide with the results reported by Borase et al. (2015) [22].

- **MR Test**

This test is performed to detect the ability of an organism to produce stable acids end products from the supplied glucose. The MR test was performed on all the seven isolates from which the isolates JNEC/AMB/02 and JNEC/LAB/01 gave positive results and the isolates JNEC/ID/01, JNEC/AMB/01, JNEC/AMB/03, JNEC/AMB/04 and JNEC/SHE/01 gave negative results.

- **VP Test**

This test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. The VP test was performed on all the seven isolates from which the isolates JNEC/AMB/03, JNEC/AMB/04 and JNEC/SHE/01Sheep gave positive results and the isolates JNEC/ID/01, JNEC/AMB/01, JNEC/AMB/02 and JNEC/LAB/01 gave negative results indicating respective ability and inability of the cultures in production of carbinol.

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

- **Citrate Utilization Test**

This test is used to determine the ability of bacteria to utilize sodium citrate as it is the only carbon source. The citrate utilization test was performed on all the seven isolates and all the isolates gave negative results for this test indicating citrate cannot be metabolized by these isolates. Our results coincide with the results as reported by Borase et al. (2015) [22].

- **Sugar Fermentation Test**

This test is used to determine whether or not bacteria can ferment a specific carbohydrate source. Three different carbohydrates namely glucose, fructose and lactose were used while performing this test. The sugar fermentation test when performed gave positive results for all the three carbohydrate sources indicating all these sugars are readily metabolizable by these isolates. Our results coincide with the results as reported by Borase et al. (2015), Kumar et al. (2015) and Pundir et al. (2013) [23,24,25].

- **Urease Test**

This test is used to determine the ability of an organism to split urea, through the production of an enzyme urease. Urease test was performed on all the seven isolates and all the isolates gave negative results for this test indicating absence of urease enzyme.

- **Milk Coagulation Test**

LABs have the property of milk coagulation by acid production. The milk coagulation test was performed on all the seven isolates and all the isolates gave positive results for this test. This result confirms that all the six isolates belong to LABs. However molecular 16s sequencing would be a confirmatory test to reveal the exact nature of the cultures. Table 5 depicts a comparative overview of the biochemical tests for all the cultures.

Table 5: Comparative overview of Biochemical Characterization of isolates

Sr. No.	Biochemical Tests	JNEC/ID/01	JNEC/SHE/ 01	JNEC/AMB/ 01	JNEC/AMB/02	JNEC/AMB/ 03	JNEC/AMB/04	Std. LAB
1.	Voges-Proskauer test (VP test)	-ve	+ve	-ve	-ve	+ve	+ve	-ve
2.	Indole test	-ve	-ve	-ve	-ve	green color observed	-ve	-ve
3.	Citrate utilization test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4.	Milk coagulation test	+ve	+ve	+ve	+ve	-ve	+ve	+ve
5.	Glucose fermentatio	+ve	+ve	+ve	+ve	-ve	+ve	+ve

IJARSE
ISSN (O) 2319 - 8354
ISSN (P) 2319 - 8346

	n test							
6.	Catalase test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7.	Oxidase test	-ve	-ve	-ve	-ve	-ve	-ve	-ve

3.6. Evaluation of Probiotic Activity

- **NaCl Tolerance test**

This test is used to determine the ability of any bacteria to grow in variable amount of sodium chloride. The NaCl tolerance test was performed on all the seven isolates using 3%, 5%, 7% and 9% concentrations of NaCl. The graph for the optical density versus the concentration of NaCl for all the seven microbial cultures is as shown in Fig. 2. It can be clearly observed that the salt tolerance of the isolate obtained from idli batter exhibited highest salt tolerance among all the cultures. All the isolates except JNEC/AMB/02, exhibited relatively more salt tolerance than the standard *Lactobacillus* culture, thereby promising a significant probiotic activity.

- **pH Tolerance test**

This test is used to determine the ability of bacteria to grow in variable pH conditions. The pH tolerance test was performed on all the seven isolates at varying pH of 3, 5, 7and 9. The graph for the optical density versus the varied pH for all the isolates obtained is as shown in Fig. 3.

- **Antimicrobial activity test**

This test is used to determine the ability of isolate to produce bacteriocins that are antimicrobial in nature. The antimicrobial activity test was performed on all seven isolates using agar well diffusion method. Antimicrobial activity of the isolates is as depicted in Fig. 4. All isolates except the isolate JNEC/AMB/03 showed zone of inhibition against *E. coli*. This could be due to inability of that culture to produce antimicrobial compounds or could be attributed to absence of antimicrobial property against *E. coli.* Generally antimicrobial production like nisin, bacteriocins is a typical feature of LABs. Many authors have found the associated antimicrobial activity of LABs [8,11,12,13,26]. In our study a significant zone of inhibition of 0.57 cm was obtained with JNEC/SHE/01 which is almost twice that of the zone of inhibition of the standard culture.

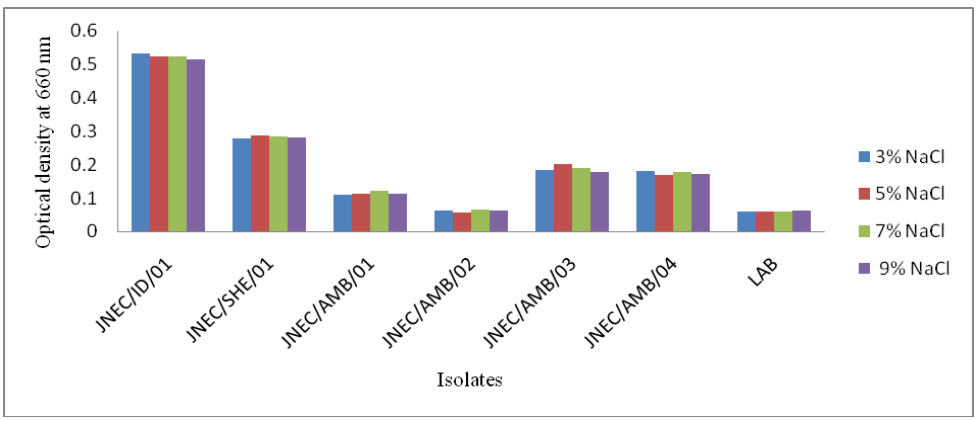

Figure 2: effect of varying salt concentration upon growth of isolates

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IJARSE
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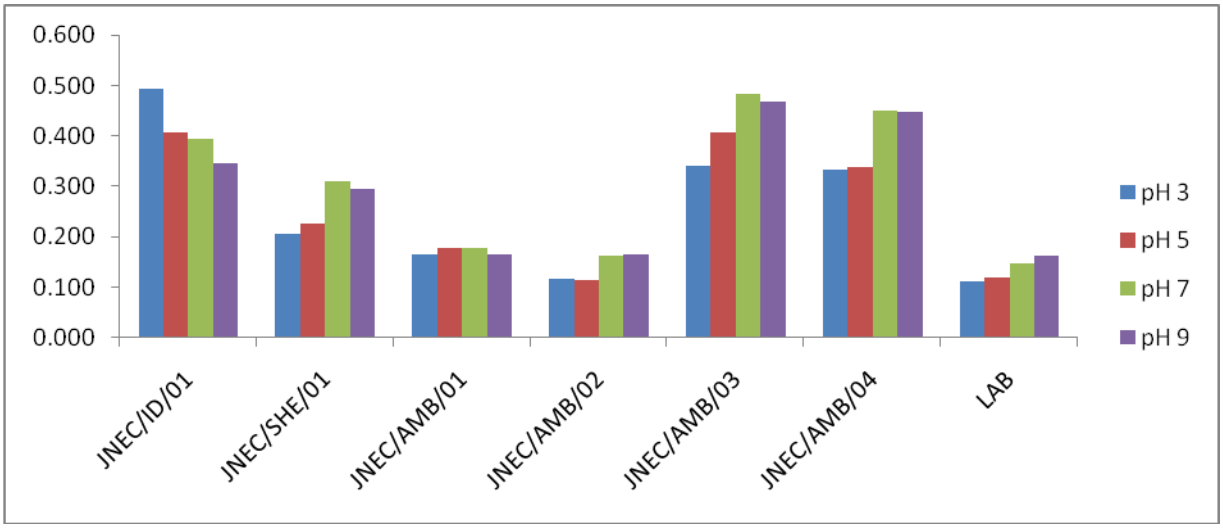
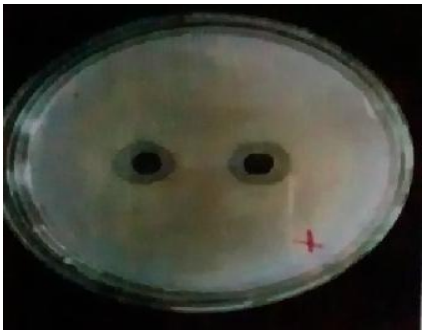


Figure 3: effect of varied pH on the growth of isolates

JNEC/ID/01 (0.285 cm)

JNEC/SHE/01 (0.57 cm)



JNEC/AMB/01 (0.33 cm)

JNEC/AMB/02 (0.23 cm)

JNEC/AMB/03 (Nil)

JNEC/AMB/04 (0.38 cm)

IJARSE
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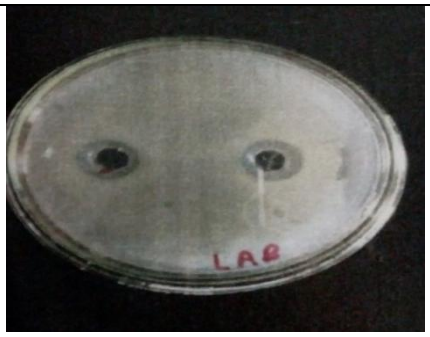

Std. LAB (0.28 cm)

Figure 4: **Antimicrobial property of isolates**

IV. CONCLUSION

Total of six different isolates were isolated from Indian Fermented Food and dairy products. When evaluated, these isolates exhibited similar characteristics of microscopic and biochemical features of LABs. However further molecular identification is essential for its species confirmation. These isolates also displayed probiotic properties when evaluated for its pH and salt tolerance. JNEC/ID/01 exhibited maximum pH as well as salt tolerance as compared to the commercial *Lactobacillus* strain, indicating its further utilization in the nutraceutical formulations.

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