

## Screening of indigenous active lactic acid bacteria isolated from freshly drawn raw milk

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### ABSTRACT

Main emphasis of the present study was to isolate and identify active Lactic Acid Bacteria from various raw milk. Totally 36 freshly drawn various raw cow, buffalo, goat and sheep milk from Tirupur and Erode region in Tamil Nadu, were collected. From the sample 56 Lactic Acid Bacteria (LAB) isolates were taken randomly. The LAB were phenotypically identified and grouped based on the morphological, physiological and biochemical study. The strain survival were also assessed under stomach acid condition like low pH and resistance to bile salt 0.3%. Antibiotic sensitivity tests were performed for five antibiotics. After hemolytic activity on blood agar medium the antibacterial activities of the isolates were tested against two pathogenic bacteria *E.coli* and *Staphylococcus sp.*, at pH 6.5 by overlay method. The tested isolates showed invitro inhibitory zone against pathogenic bacteria. From this study we can conclude that raw milk is good source of active lactic acid bacteria. Out of the 56 LAB isolates 28 exhibited good probiotic properties and potentiality was characterized in future.

**KEY WORDS:** RAW MILK, LACTIC ACID BACTERIA, PHENOTYPICAL IDENTIFICATION, ANTIBIOTIC SENSITIVITY, ANTIBACTERIAL ACTIVITY

### INTRODUCTION

Ancient Indian has practice to consume freshly drawn raw milk without boiling. They believed that raw milk has some good property. Milk is an excellent medium to carry an active lactic acid bacteria and buffering capacity of milk helps to improve the survival of pro-

biotic flora in the gastrointestinal tract, (Kailasapathy and Phillips 2008). Lactic acid bacteria tolerate high salt concentrations as its allows the bacteria to begin metabolism, which produces acid that further inhibits the growth of undesirable microorganisms (Pooja Thakkar *et al.*, 2015). Probiotic LAB can be a suitable alternate of antimicrobial agents and recently found to play a

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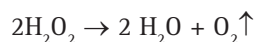
positive role in mental health. Milk is one of the natural habitats and rice source of LAB, (Delavenne *et al.*, 2012, Wouters *et al.*, 2002 and Misganaw wassie and Teketay wassie, 2016, Mokoena *et al.*, 2016).

The LAB in milk and milk products enhance bioavailability of nutrients and act as a preservative (Misganaw wassie and Teketay wassie, 2016). Fermented and functional foods and the products are crucial to the human health (oktay Yerlikaya, 2014). Potential probiotic isolates of *Lactobacillus rhamnosus* and *L. plantarum* were present in indigenous goat milk (Setyawardani *et al.*, 2011). The probiotic *L. yoghurt* supplementation to worldwide waterborne diarrhea causing Giardia infected mice reduced the severity of Giardia infection (Geeta shukla *et al.*, 2010). Lactic acid bacteria produce antimicrobial compounds, vitamins or useful enzymes which could help in promotion of food industry (Ashmaig *et al.*, 2009). Identify potent attributes to meet out current demands of the functional food industry (Subhashini, 2014).

## MATERIALS AND METHODS

36 samples of raw fresh milks were collected from lactating cow, buffalo, goat and sheep in the rural area surrounding of Tirupur & Erode District. Samples were collected using sterile centrifuge tubes and stored in an icebox until delivery of the laboratory for analysis. Till the analysis samples were kept in 4°C (refrigerator). About 1ml of milk sample was mixed with 9ml of saline [8.5g / L] to make an initial dilution [10<sup>-1</sup>]. The suspension was used for making suitable serial dilutions up to 10<sup>-8</sup>. Enumeration of LAB was determined using MRS (Man de Rogosa Sharpe) agar and M17 agar medium by pour plate [1ml in 15ml medium] incubated at 37 °C for 24-48 hours. After incubation colonies were chosen based on their morphology on MRS (pH-5.7) agar plate. The typical LAB were randomly picked up and purified for further work. Simple tests such as gram staining, catalase test, motility and sugar fermentation test were performed for isolates.

The isolates grown in freshly prepared liquid media and incubated overnight. After incubation the cells were taken and then gram staining procedure was performed. The gram reaction of the isolates was determined by light microscopy. Catalase enzyme produced by many microorganisms that breaks down the H<sub>2</sub>O<sub>2</sub> into water and oxygen that releases O<sub>2</sub> gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.



The freshly grown liquid cultures were also used for catalase activity by dropping 3% hydrogen peroxide

solution onto 1 ml of overnight cultures and their catalase activity was observed.

(Thakkar *et al.*, 2015). MRS broth supplemented with different Sugars (glucose, lactose and maltose) and phenol red as pH indicator was inoculated with active cultures at 1%, incubated at 37°C for 24 hours. The cultures were identified based on acid and gas production in Durham's tube after the incubation period. To check the growth of isolates at various pH, MRS broth supplemented with different pH 2.0, 3.0, 7.0, 8.5 was prepared, 1% of fresh culture was inoculated and then incubated at 37°C for 28 hours. During incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth. Turbidity also measured at 620nm. Overnight active cultures were inoculated at 1% in MRS broth tubes and incubated up to 7 days at 15, 37, 45 and 55°C. Extent of growth was visually recorded based on intensity of turbidity. Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of Na Cl viz. 3.5, 6.5 and 18% (w/v) along with their respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

Blood hemolysis test was carried out as per the method of Mabrouk *et al.*, (2014). As the strains were isolated from food material, blood haemolysis test was performed, to eradicate any chance that our isolates may be pathogenic. It is also one of the criteria for assessing the safety of use of probiotics as food supplements. Pathogens produce highly toxic substance which lyse the RBC and forms a clear zone around them. The haemolytic activities of isolated strains were determined according to (Marakoudakis *et al.*, 2009) as follows: all examined strains were grown in MRS broth at 37°C for 24 hours and then streaked onto Columbia agar base plates supplemented with 5 % (v/v) whole human blood. The plates were incubated at 37 °C for 48 hours. Then the clear zones and the color of haemolysis around the growth colonies were observed. Antibiotic susceptibility test was done using the method of Singh *et al.*, (2014).

Probiotic strains must be sensitive towards the antibiotics. There is a light risk that antibiotic resistance probiotic strain may transfer the antibiotic resistance genes to the pathogens via transformation in the gut. Due to any chance resistant pathogens get introduced into the human via food chain and cause serious problems. Sensitivity of probiotics strains towards the antibiotics being tested by using Kirby - Bauer disc diffusion technique. Tetracycline, Penicillin, Vancomycin, Strep-

tomycin and Kanamycin was used. For this process MRS agar inoculated with LAB and disc were placed. After the incubation period (24 hours/ 37°C) and inhibition zones were observed to determine the antibiotic resistance of isolates. Antagonistic activity was carried out by the method of (Bolanle *et al.*, 2015).

The agar overlay method was employed to determine the ability of the viable lactic acid bacteria strains to inhibit the growth of the indicator pathogens, *E.coli* and *Staphylococcus aureus*. A loop full of LAB in MRS broth was inoculated on MRS agar plate as a thick line of about 2mm and about 30mm long at a good away from the edge of the plates and incubated under microaerophilic condition at 37°C for 24 hours. After incubation, the MRS agar plates were overlaid with approximately  $0.2\text{ml} \times 10^{-7}$  CFU /ml of an overnight broth culture of the test pathogens inoculated in 10ml of Muller Hinton soft agar (with 0.7% agar-agar). The overlay was allowed to set and incubated at 37°C under aerobic condition. The plates were then examined for zone of inhibition around the line of the LAB and the clear zones were measured.

## RESULTS AND DISCUSSION

A total of 56 LAB isolates were identified from various freshly drawn raw milk samples collected from surrounding of Tirupur and Erode district (Table 1).

All the fifty six isolates were gram positive, non-motile in hanging drop method, fifty isolates were catalase negative and only six showed positive to catalase test were not a LAB. The cell morphology of fifty six isolates was evaluated through grams reaction microscopic observation and majority of 36 were found to be rods and the remaining of 20 isolates were cocci shaped. Among the isolates 42 were able to produce CO<sub>2</sub> from glucose fermentation. This result showed that they were

heterofermentative and remaining of 14 isolates were homofermentative. Homofermentative LAB utilize glucose via EMP pathway and heterofermentors utilize via HMP (hexose monophosphate pathway) described by Rattanachaikunsopon and Phumkhachorn (2010). All the isolates grew at 37°C but only 21 isolates were grew at 15°C; in 45°C and 55°C, only ten isolates were showed limited growth. In 3.5 % Na Cl concentration all the isolates grew well and only 27 showed growth at 6.5% Na Cl concentration. 21 isolates showed limited growth at 18% Na Cl concentration.

Based on the morphological, physiological and biochemical test chemometric result hierarchical cluster analysis (AHC) for finding homogenous group of isolates and graphics were performed using XLStat software version 2018.5, Addinsoft. Dendrogram showing the similarity relationship among the 56 isolates of LAB obtained from various raw milk. Similarities were calculated by the simple matching coefficient and grouping was performed by Agglomeration hierarchical cluster (AHC) analysis using Un-weighted pair group average linkage analysis. A total of 56 isolates characterized using set of 17 phenotypic test. An abridged dendrogram depicting the similarity relationship among the isolates were divided into six groups shown in figure 2. While the phenotypic characterization of the main clusters were summarized in linkage analysis figure 1.

Results showed that all the examined strains did not exhibited  $\beta$  haemolysis and most of the strains were  $\gamma$  haemolytic (non-pathogenic), while seven exhibited  $\alpha$  haemolysis. Out of seven, five isolates were catalase positive there were not a LAB avoided of further work. Some of Enterococcus showed  $\alpha$  haemolysis and as well as  $\beta$  haemolysis summarized by W.Liu *et al* (2014). According to the European Food Safety Authority (2012) antibiotics of Tetracyclin, Streptomycin and Kanamycin were used for antibiotic susceptibility testing of the isolated LAB.

Table 1. Origin and designation of the isolates of Lactic acid bacteria

S. No	Sample	Region	No. of samples	No. of isolates	Designation of Strains
1	College	Tirupur, Erode	2	5	C2, C9, C16, C7, C19
2	Sindhu	Tirupur, Erode	11	11	C3, C5, C11, C13, C14,C17, C18, C20, C21, C22, C23
3	Cooli	Tirupur, Erode	2	5	C1, C6, C8, C12, C24
4	Jersey	Tirupur, Erode	1	3	C4, C10, C15
5	Buffalo	Tirupur, Erode	2	5	B25, B26, B27, B28, B29
6	Goat (Black)	Tirupur, Erode	4	8	G4,G15,G16,G21,G25,G26,G27,G1
7	Goat (white)	Tirupur, Erode	12	17	G2,G6,G13,G14,G5,G10, G7,G8,G12,G17,G9,G18, G19,G20,G22,G23,G24
8	Sheep	Tirupur, Erode	2	2	S3,S11

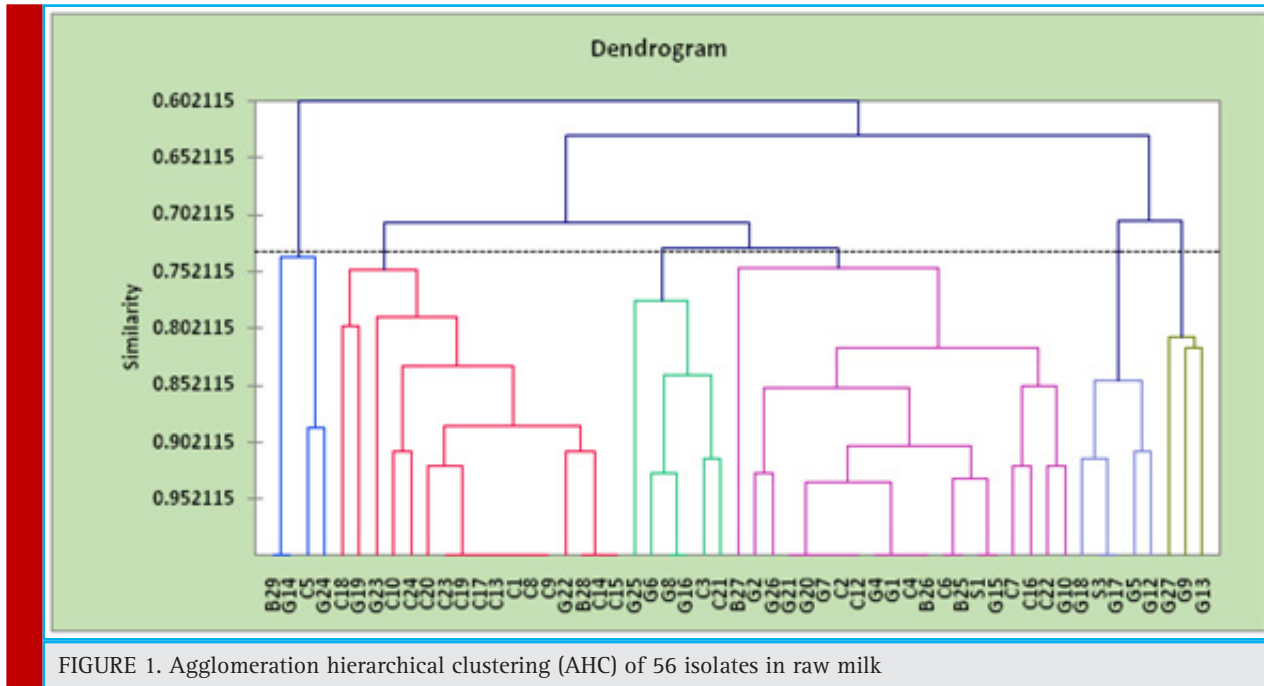


FIGURE 1. Agglomeration hierarchical clustering (AHC) of 56 isolates in raw milk

Results showed in figure 3 describes 50% of the isolates were resistant to antibiotics. A similar observation was given by Hawaz (2014) who also noticed Ethiopia curd had *Lactobacillus* and *L. delbruekii*, *L. brevis* and *L. casei* were resistance to antibiotic streptomycin and gentamycin; *L. fermentum*, *L. lactis* and *L. rhamnosus* were resistant to streptomycin. *L. leichmanni*, *L. acidophilus* and *L. coagulans*, which were only sensitive to antibi-

otics. Furthermore Pundir et al (2013) had reported LAB isolates from food sample were found to be resistance to most of the antibiotics. Zarour et al (2012) had also reported goat and camel milk isolates were resistant to Vancomycin the strains had a poly resistance to antibiotics which is attributed to plasmid transposons in many bacterial strains. Based on the frequency of antibiotic sensitive isolates were taken for further analysis.

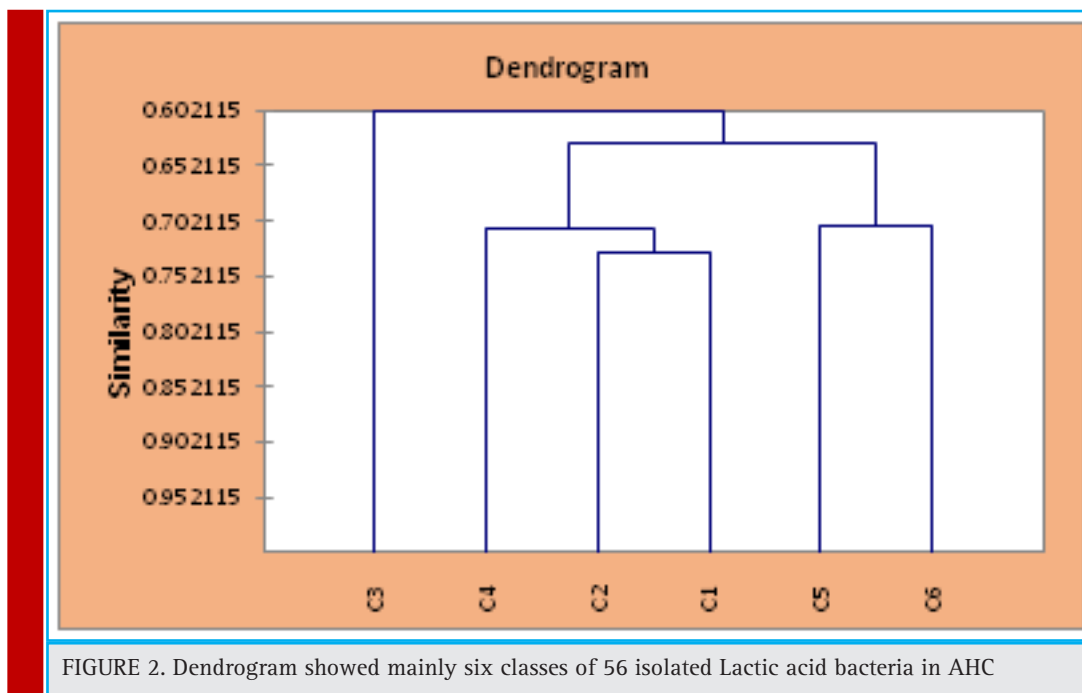


FIGURE 2. Dendrogram showed mainly six classes of 56 isolated Lactic acid bacteria in AHC

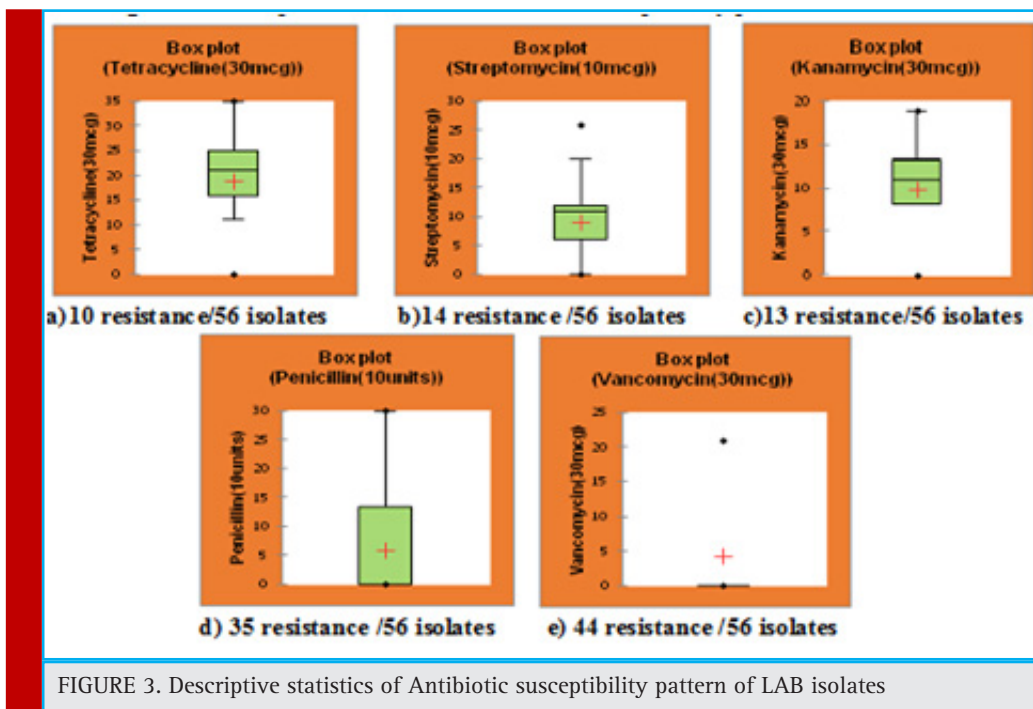


FIGURE 3. Descriptive statistics of Antibiotic susceptibility pattern of LAB isolates

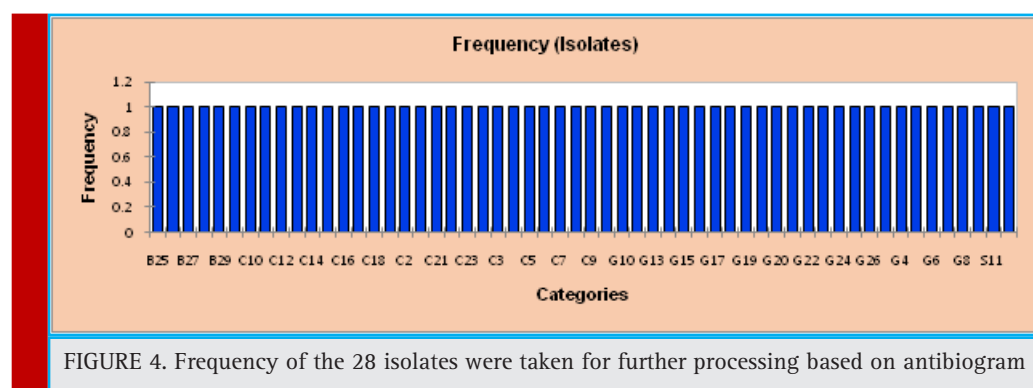


FIGURE 4. Frequency of the 28 isolates were taken for further processing based on antibiogram

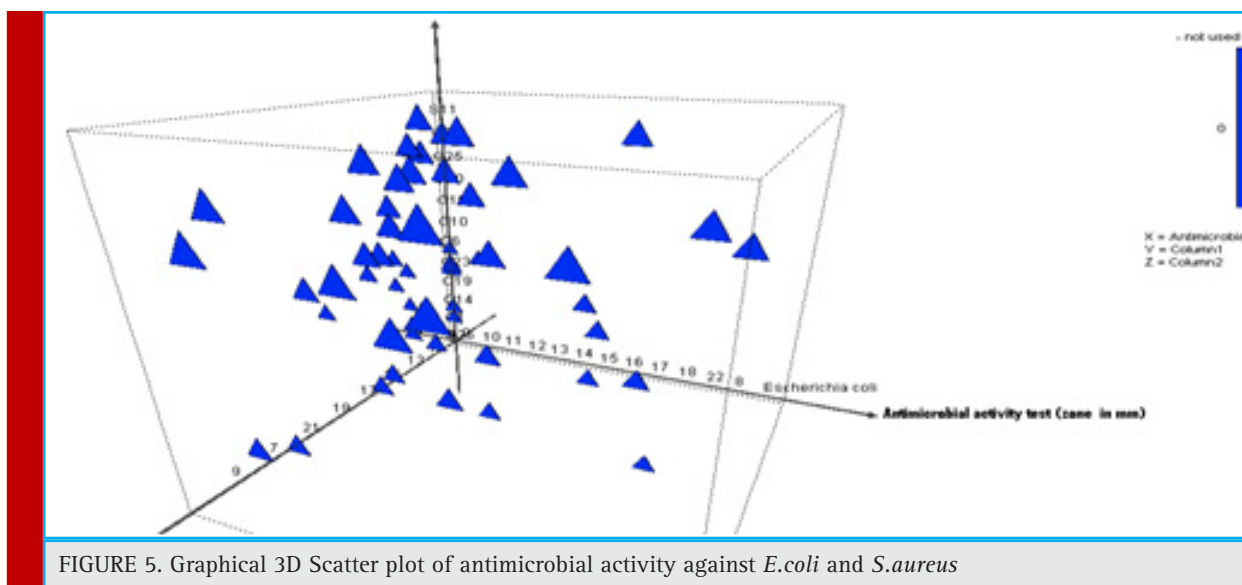


FIGURE 5. Graphical 3D Scatter plot of antimicrobial activity against *E.coli* and *S.aureus*

Table 2. Blood haemolysis, Antibiogram and antimicrobial activity of LAB isolates

S. No	Isolates	Blood haemolysis test			Antibiotic susceptibility test (zone in mm)					Antimicrobial activity test (zone in mm)	
		$\alpha$	$\beta$	$\gamma$	Tetracycline (30 mcg)	Penicillin (10 units)	Vancomycin (30 mcg)	Streptomycin (10 mcg)	Kanamycin (30 mcg)	E. coli	S. aureus
1	C1	-	-	-	R	R	R	R	R	22	19
2	C2	-	-	-	22	R	R	9	14	-	13
3	C3	-	-	-	25	10	R	12	12	-	14
4	C4	+	-	-	23	R	R	10	8	-	19
5	C5	-	-	-	R	R	R	R	R	-	13
6	C6	+	-	-	15	R	R	10	11	-	-
7	C7	-	-	-	29	R	R	12	11	11	11
8	C8	-	-	-	31	R	R	13	14	-	16
9	C9	-	-	-	23	9	R	12	12	-	14
10	C10	-	-	-	R	R	R	R	R	14	18
11	C11	-	-	-	R	R	R	R	R	-	-
12	C12	-	-	-	17	13	17	12	13	16	14
13	C13	-	-	-	22	20	R	11	12	-	13
14	C14	-	-	-	R	R	R	R	R	-	-
15	C15	-	-	-	31	R	R	12	12	-	13
16	C16	+	-	-	19	R	R	11	12	-	12
17	C17	-	-	-	22	15	R	11	11	-	12
18	C18	-	-	-	33	R	R	12	12	18	16
19	C19	-	-	-	32	10	R	16	15	14	21
20	C20	-	-	-	27	14	R	13	11	16	13
21	C21	-	-	-	22	13	R	10	11	14	18
22	C22	-	-	-	27	12	R	11	11	15	11
23	C23	-	-	-	32	21	R	26	11	-	-
24	C24	-	-	-	21	22	13	20	17	-	-
25	B25	+	-	-	18	R	19	12	13	-	22
26	B26	+	-	-	R	R	R	8	10	-	16
27	B27	+	-	-	23	R	R	13	16	-	15
28	B28	-	-	-	21	R	R	10	11	-	11
29	B29	-	-	-	31	R	21	11	12	-	8
30	G1	-	-	-	R	R	R	R	R	-	20
31	G2	-	-	-	R	R	R	R	R	-	14
32	S3	-	-	-	35	19	19	13	14	13	17
33	G4	-	-	-	22	R	R	9	11	-	11
34	G5	-	-	-	34	14	20	15	18	10	9
35	G6	-	+	-	21	R	R	10	8	-	12



36	G7	-	-	-	21	R	R	15	16	-	-
37	G8	-	-	-	22	R	R	10	10	11	19
38	G9	-	-	-	17	16	16	13	14	16	-
39	G10	-	-	-	16	R	R	R	R	-	15
40	S11	-	-	-	24	R	R	R	17	-	11
41	G12	+	-	-	30	14	16	11	12	-	16
42	G13	-	-	-	21	30	20	14	18	14	8
43	G14	-	-	-	30	17	R	13	13	13	15
44	G15	-	-	-	16	R	19	11	13	8	10
45	G16	-	-	-	22	R	R	11	11	-	14
46	G17	-	-	-	18	15	20	13	17	15	8
47	G18	-	-	-	14	R	R	11	12	12	7
48	G19	-	-	-	25	14	R	R	19	10	-
49	G20	-	-	-	17	R	R	12	14	17	20
50	G21	-	-	-	16	R	R	R	R	-	17
51	G22	-	-	-	24	R	20	R	R	-	9
52	G23	-	-	-	R	R	R	R	R	-	-
53	G24	-	-	-	21	R	R	11	10	-	13
54	G25	-	-	-	R	R	R	R	R	-	12
55	G26	+	-	-	11	10	R	10	11	14	22

In this study, the antimicrobial activity showed 46 isolates produced maximum inhibition zone against bacterial pathogen *Staphylococcus* sp., comparatively less isolates of 22 produced zone against *E.coli* sp. Showed in figure 4. The present work contrary with Hawaz (2014) had reported *Lactobacillus* strains showed antibacterial effect against the pathogenic *E.coli*, *Staphylococcus* sp., and *Salmonella thyphimurium*. This may vary from type of strain and class of bacteriocin produced by LAB.

## CONCLUSION

The present study concluded that freshly drawn animal milk had rich source of lactic acid bacteria and predominant 64% *Lactobacillus* sp. and 34% of isolates were coccus that tentatively had *Enterococcus*, *Leuconostoc*, *Streptococcus*, *Lactococcus* and *Pediococcus* which is in conformity with earlier work. As raw milk is used more in Tamil Nadu being consumed frequently and occasionally, consumers received significant amount of LAB. Potentialities of probiotic characteristics are being done in further studies. Indeed, a natural preservation of characterized LAB in under investigation. Further work is required on species level identification of isolates and bioactive compounds produced by LAB.

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