WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 5, 599-612. Research Article ISSN 2277 – 7105

ISOLATION, IDENTIFICATION AND CHARACTERISATION OF A NOVEL PROBIOTIC STRAIN (LACTOBACILLUS PARACASEI KUMBB005) FROM COW MILK SAMPLES AND ITS ANTIBACTERIAL ACTIVITY

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Article Received on
10 May 2014,

Revised on 05 June 2014,
Accepted on 29 June 2014

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ABSTRACT

Lactobacillus sp., was isolated from 10 raw cow milk samples using MRS agar, out of 15 isolates 4 *Lactobacillus* isolates (KUMBB001, KUMBB002, KUMBB003, KUMBB005) were selected for characterization study. The isolates were examined for their probiotic properties such as pH resistance, NaCl tolerance, bile salt tolerance, antibacterial activity and antibiotic susceptibility. Among the 4 isolates KUMBB005 showed resistance to acidic pH 6, maintained good growth at NaCl concentration (1-9%) and the isolates depicted good tolerance against (1-5%) bile salt (Ox-gall). Based on the biochemical characterization the isolates KUMBB005 has shown potent growth and antibacterial activity which was further subjected to 16S rRNA sequencing. The sequence was submitted in NCBI with accession number KM008572. After sequencing and phylogenetic analysis the isolate KUMBB005 was identified as *Lactobacillus parcasei*. The cell free extract of the isolate exhibited good antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* MTCC 4030, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* MTCC 734. *Lactobacillus paracasei* KUMBB005 also exhibited good resistance towards various antibiotics such as Kanamycin, Erythromycin, Vancomycin and Streptomycin. *Lactobacillus paracasei* KUMBB005 obtained from milk sample were more effective than other isolates and could be assigned as potential strain for further research work towards its probiotic application.

KEY WORDS: *Lactobacillus* sp., Characterization, 16S rRNA sequencing, *Lactobacillus paracasei*, Antibacterial activity.

INTRODUCTION

In the history since 1900, the start of Industrial microbiology, interest in microorganism from food source is increased due to the potential of new bacterial species and strains [1]. Among various food sources milk plays a major role as nutritious for humans and animals, addition to nutritional benefits the presence of specific components or beneficial bacteria called probiotic is gaining scientific credibility at a rapid pace [2]. *Lactobacillus* is one of the most important genera of Latic acid bacteria; LAB is widely distributed and occurs as an indigenous microflora in raw milk that plays an important role in many food and feed fermentation [3]. LAB are commonly used in most probiotics preparations due to the historical belief that they are desirable members of the intestinal microflora and are thus constructively amend the balance of intestinal microflora, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection [4]. Genus *Lactobacillus* consists of a genetically and physiologically diverse group of rod-shaped Gram positive, non-spore forming, nonpigmented, catalase negative and microaerophilic organism considered as generally recognized as safe (GRAS) organisms [5]. These bacteria are exploited in the field of fermented food production, medical and veterinary application. In recent years, active investigation in the field of probiotics, due to its bacteriocinogenic property by producing antimicrobial substances such as bacteriocin, H_2O_2, CO_2, and diacetyl (2,3 butanedione) have a great potential to be used in therapeutics and as food bio-preservatives. It is considered as safe with great economic importance and it exerts a spectrum of antibacterial activity against various organisms [6, 7, 8, 9, 10]. In view of exceeding facts the present study was to isolate and characterize *Lactobacillus* sp., from raw cow milk samples and also to study its metabolism towards antibacterial activity.

MATERIALS AND METHODS

Isolation and identification

10 fresh raw cow milk samples were collected from the local area of Coimbatore under aspectic condition, cold stored and transferred to the laboratory within 2 h. de Mann Rogosa Sharpe (MRS) agar were used for isolation of *Lactobacillus* sp.,. The milk samples were homogenized with saline using mortal and pestel, serially diluted and pour plated on MRS plates and incubated at 37°C, under anaerobic conditions (11). The isolates were studied

macroscopically for their shape, colony morphology and confirmed microscopically by performing Gram staining.

Biochemical characterization

The biochemical tests performed were Indole, Methyl Red (MR), Voges Proskauer (VP), Oxidase, Catalase tests and growth at 35º C - 40º C as described previously [12]. Carbohydrate utilization potential of the isolates was also tested by inoculating 0.1 mL of inoculum in 5mL of MRS broth containing Glucose, Xylose, Sucrose, Fructose, Lactose, Maltose, Ribose, Rhamnose, Mannitol and Dextrose. Tubes were, incubated at 37°C for 24 h and the results of color change were recorded as positive or negative.

Effect of pH tolerance

Acidification was measured by change in pH. The inoculum was inoculated to MRS broth adjusted with various pH ranges (1-8) with conc. NaOH (10N) and conc. HCl (16N) according to previous study [13]. After 24h of incubation the cell density of bacterial isolates was measured using a spectrophotometer at 600 nm.

NaCl Tolerance

The isolates were grown in MRS broth having different NaCl concentration (1-9%) and incubated at 37°C for 24 h as described earlier [14]. The culture tubes were observed for the presence or absence of growth. After 24 h incubation, growth was determined using a spectrophotometer reading the optical density at 600 nm.

Bile salt tolerance

Bile salt tolerance of the isolates was investigated by determining their growth in MRS broth containing different levels (1, 2, 3, 4 and 5%) bile salts (Ox-gall) according to the reports [15]. Optical densities were measured using a spectrophotometer at 600 nm after 24 h of incubation.

Molecular identification

Following the biochemical characterization of the 4 isolates, the isolate showing precise characteristic alone were subjected to species level identification by 16S rRNA sequencing. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the nr database of NCBI genbank database. Based on maximum identity score first ten

sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA.

Antibacterial activity

Antimicrobial activity of cell free supernatant was checked against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* MTCC 4030, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* MTCC 734. Cell suspensions of the indicator microorganisms were prepared on nutrient broth and the turbidity was compared to 0.5 McFarland standards. Muller Hinton Agar plates were prepared and lawn cultures of indicator microorganisms were made by swab inoculation. The well with the holding volume of 50 µL was made in the center of the plate using well cutter. 24h old cultures of isolate were centrifuged at 12,000 x g for 10 min, and 50 µL of the supernatant was loaded in the well and the plates were incubated at 37º C for 24 h. The antibacterial activity was determined and zone of inhibition was measured in millimeter (mm).

Antibiotic susceptibility

The antibiotic susceptibility was determined towards antibiotics, namely Kanamycin, Erythromycin, Vancomycin and Streptomycin. The selected isolate were inoculated in MRS broth at 37°C for 24 h. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the MRS agar plate. Susceptibility of the isolates to antibiotics was performed by the disc diffusion method; the antibiotic disc was placed on the surface of the agar plates, precaution was taken to ensure that there was uniform contact between the antibiotic disc and agar plate. The plates were then incubated at 37°C for 24h.

RESULTS

Isolation

In the present study *Lactobacillus paracasei* was isolated from the 10 raw cow milk samples collected from in and around Coimbatore. From the 10 samples 4 bacterial cultures was isolated, from de Mann Rogosa Sharpe (MRS) agar, based on morphology, cultural and biochemical characterization the bacterial isolates were selected and named as *Lactobacillus* sp., KUMBB001, KUMBB002, KUMBB003 & KUMBB005 respectively and were grow on selective MRS agar media. All the 4 isolates produced round shape, off-white to cream colour, shiny colonies on MRS agar. The microscopic observation of the 4 isolates at 100x resolution shows Gram positive, small, single or clump rods (Fig.1).

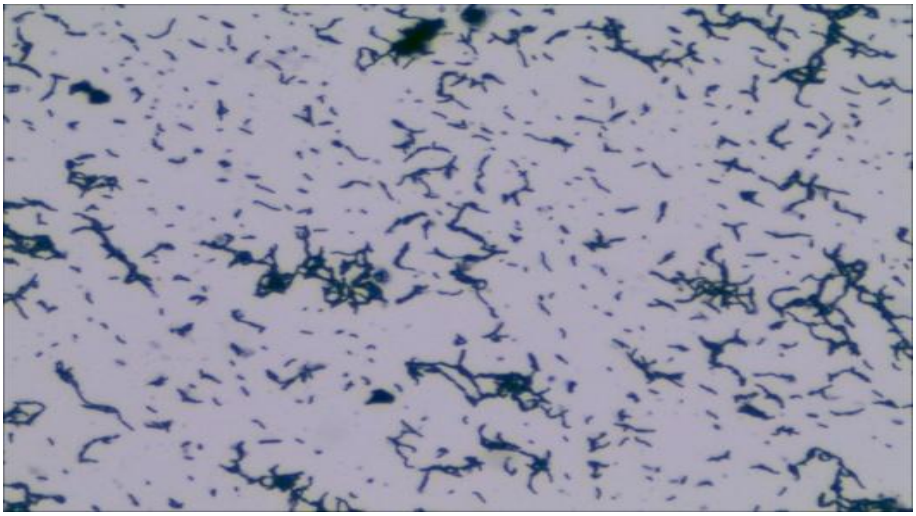

Fig.1. Microscopic observation of *Lactobacillus* sp.,

Biochemical characterization

Growth at 35°C - 40°C, the isolates showed good growth at 37°C whereas the isolates could not grow at low and elevated temperature. The isolates showed negative for Indole, MR, VP, Oxidase and Catalase. Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to the bio-oxidation of a substrate, frequently a carbohydrate. Therefore in this study the isolates were subjected to different sugars to determine its fermentation profile. All isolates were able to ferment Glucose, Xylose, Sucrose, Fructose, Lactose, Maltose, Ribose, Rhamnose, Mannitol, and Dextrose. It indicates that the 4 isolates are able to grow in utilizing different type of carbohydrates (Table 1). The authors reported catalase test as an important identification factors for *Lactobacillus* sp.,. Catalase, an extracellular enzyme helps in degradation of hydrogen peroxide that generates effervescence [18] and therefore the absence of effervescence is taken as negative for catalase enzyme production. All the isolates showed negative indication for catalase test is akin to results reported previously [2]; thereby its presence or absence in a microbial cell can be used as a significant diagnostic tool.

Table.1. Showing the biochemical characteristics of isolates.

S. No.	Test parameter	Isolates			
		KUMBB001	KUMBB002	KUMBB003	KUMBB005
1	Indole	-	-	-	-
2	MR	-	-	-	-
3	VP	-	-	-	-
4	Oxidase	-	-	-	-
5	Catalase	-	-	-	-
6	Glucose	+	+	+	+

7	Xylose	+	+	+	+
8	Sucrose	+	+	+	+
9	Fructose	+	+	+	+
10	Lactose	+	+	+	+
11	Maltose	+	+	+	+
12	Ribose	+	+	+	+
13	Rhamnose	+	+	+	+
14	Mannitol	+	+	+	+
15	Dextrose	+	+	+	+

Effect of pH

The acidic tolerance of all the 4 isolates was determined by growing the isolates from different pH range (1 to 8). Maximum growth of *Lactobacillus* sp., KUMBB005 at the pH 6 (OD 0.743±0.004) and moderate growth at pH 5 (OD 0.613±0.0005) whereas pH 7 (OD 0.307±0.005) and 8 (OD 0.185±0.003) showed reduced growth level (Fig. 2).

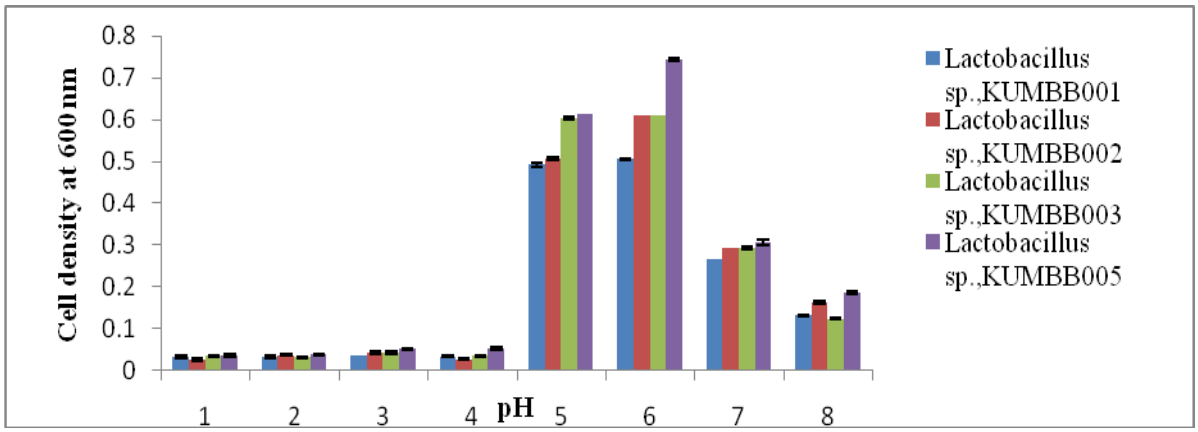

Fig. 2. Effect of pH on cell density of the isolates

Results are presented as mean ± SD of triplicate

NaCl Tolerance

Previous studies reported that NaCl tolerance is the important physiological parameter for growth of a cell as the physiological saline could prevents the cell from osmotic shock [2]. Hence the tolerance ability of *Lactobacillus* sp., was studied by subjecting them to grow from 1-9 % concentration NaCl in the growth media. All the 4 isolates maintained significant growth up to 1-4% concentration NaCl and stable growth pattern was observed from 5-9% of NaCl. Whereas *Lactobacillus* sp., KUMBB005 maintained highest growth compared to other isolates (Fig. 3).

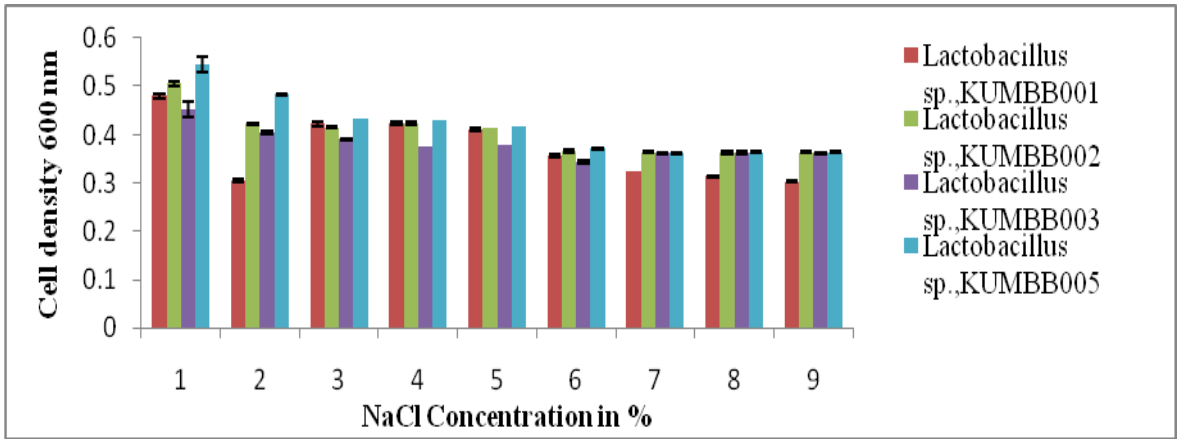

Fig. 3. Effect of NaCl on cell density of the isolates

Results are presented as mean ± SD of triplicate

Bile Salt Tolerance

The bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract such as bile salts [10]. Bile tolerance is an important factor for the survival and growth of *Lactobacilli* in the intestitinal tract; furthermore this attribute has been correlated with the hypocholesteromic effect in human [9]. The resistance ability of all the 4 isolates to bile salts was revealed after 24h of incubation at 37°C. It is found that all the *Lactobacillus* sp., maintained good growth at 1-5% of bile salt (Ox-gall) concentrations, whereas *Lactobacillus* sp., KUMBB005 reveal slight increased growth compare to all other isolates at all concentration (Fig. 4). Earlier studies reported that the resistance ability is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile [16]. The bile tolerance test was considered as important tool for characterizing *Lactobacillus*.

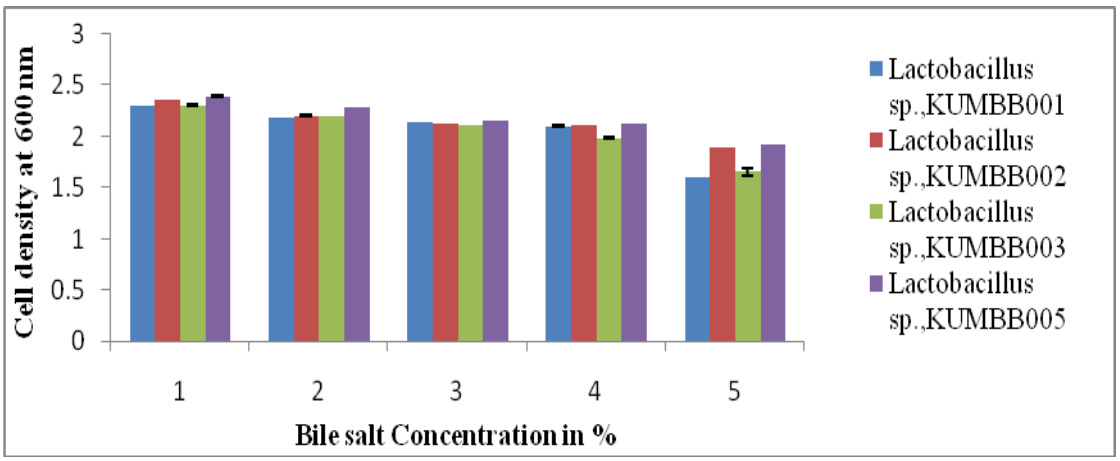

Fig. 4. The effect of Bile on cell density of the isolates

Results are presented as mean ± SD of triplicate

Molecular identification

Subsequent genetic level identification of the 4 selected bacterial isolates, KUMBB005 was found to be more dynamic and complete the characteristics of *Lactobacillus* sp., therefore KUMBB005 strain was subjected to 16S rRNA sequencing for its species level identification. DNA was isolated from the culture, its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rRNA gene was amplified by PCR. A single discrete PCR amplicon band of 1253 bp was observed when resolved on agarose. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SF and 16SR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Based on the 16S rRNA sequencing nucleotide homology and phylogenetic analysis the isolate *Lactobacillus* sp., KUMBB005 was confirmed to be *Lactobacillus paracasei*, showing 98% similarity to *Lactobacillus paracasei* strain AB5 (NCBI GenBank Acc No. JQ680428) (Table 2). Phylogenetic tree was constructed with the sequences of the PCR product and was compared with *Lactobacillus paracasei*, and the sequence was submitted in NCBI with Gen Bank Acc No. KM008572 (Fig. 5).

Table 2: Sequences producing significant alignments

Description	Max score	Total score	Query coverage	E	Max identity	Accession No
Lactobacillus paracasei strain AB5 16S ribosomal RNA gene, partial sequencegene, partial sequence	2191	2191	86%	0.0	98%	JQ680428
Lactobacillus paracasei strain IMAU62198 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KF149836.1
Lactobacillus paracasei subsp. paracasei strain FQ005 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KF418817.1
Lactobacillus paracasei strain M1-10 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KF030745.1
Lactobacillus paracasei strain E1-3 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KF030739.1
Lactobacillus paracasei subsp. paracasei strain TW11-3 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KJ026589.1

Lactobacillus paracasei subsp. paracasei strain TW16-4 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KJ026601.1
Lactobacillus paracasei strain J 21 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KJ561346.1
Lactobacillus paracasei strain L25 16S ribosomal RNA gene, partial sequence	2185	2185	86%	0.0	98%	KJ508201.1

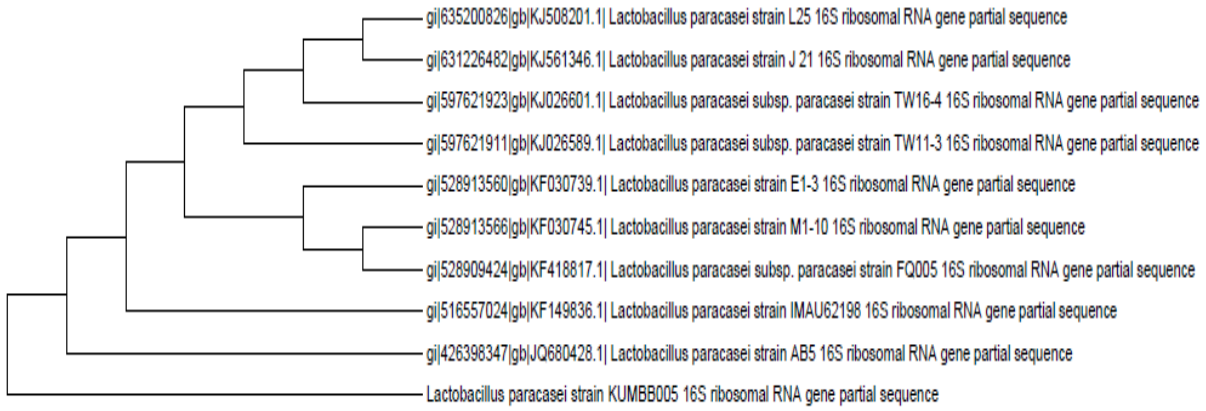

Fig. 5. Phylogenetic analysis of *Lactobacillus* sp.,

Antibacterial activity

Lactobacillus may incur antimicrobial effect by producing some substances such as organic acids, lactic, acetic, propionic acids, carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances such as bacteriocins, which may be continuously excreted by the colonies to generate the inhibitor activity against the indicator organism [8,18,19]. Following the 16S rRNA sequencing cell free supernatant of *Lactobacillus paracasei* KUMBB005 was examined for its antibacterial activity against bacterial pathogens which is considered to be important property of *Lactobacillus.* The antibacterial activity was performed by agar well diffusion method against the indicator microorganisms *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* MTCC 4030, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* MTCC 734. Researchers have reported that naturally occurring antimicrobials produced by certain bacteria are effective at controlling undesirable microorganisms. As similar to earlier reports [17, 23] the antibacterial activity of *Lactobacillus paracasei* KUMBB005 shows inhibitory zone of 21mm against *Staphylococcus aureus* ATCC 25923, 18 mm against *Escherichia coli* ATCC 25922, 18mm against *Klebsiella pneumoniae* MTCC 4030, 23mm against

Pseudomonas aeruginosa ATCC 27853, and 22mm against *Salmonella typhi* MTCC 734 (Fig. 6, Table 3). The microbiota from milk is efficient in inhibiting the pathogenic organism, will act as a barrier by developing its antimicrobial activities in the host system of defense and the inhibitory spectrum of the antimicrobial substance therefore it has a potential application as a biopreservative in food industry [9,20,21].

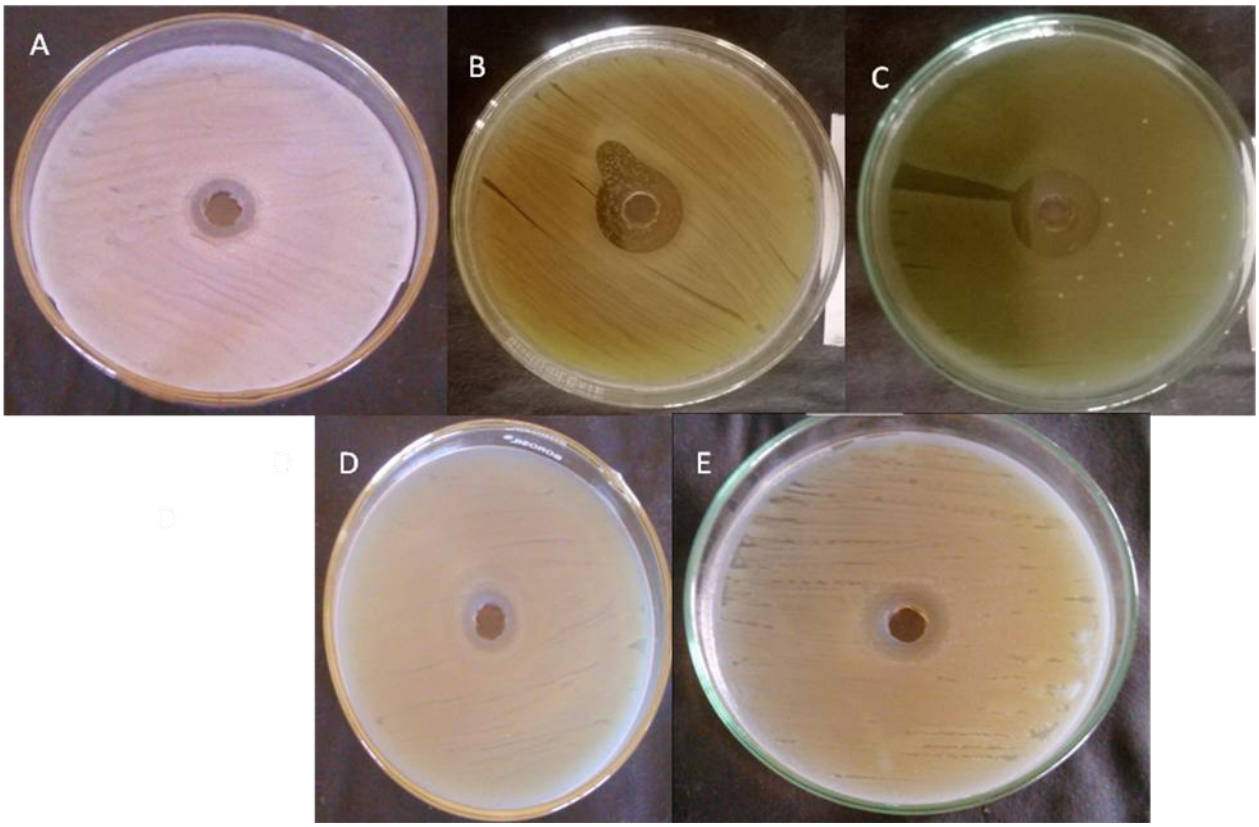

Fig. 6. Antibacterial effect of *Lactobacillus paracasei*KUMBB005.
***A - Staphylococcus aureus* ATCC 25923, B-*Escherichia coli* ATCC 25922, C- *Klebsiella pneumoniae* MTCC 4030, D - *Pseudomonas aeruginosa* ATCC 27853, E- *Salmonella typhi* MTCC 734.**

Table 3. Showing Antibacterial activity of *Lactobacillus paracasei* KUMBB005

S.No	Indicator organism	*Lactobacillus paracasei* KUMBB005 Zone of inhibition in (mm)
1	*Staphylococcus aureus* ATCC 25923	21
2	*Klebsiella pneumoniae* MTCC 4030	18
3	*Pseudomonas aeruginosa* ATCC 27853	23
4	*Escherichia coli* ATCC 25922	18
5	*Salmonella typhi* MTCC 734	22

Antibiotic susceptibility

Antibiotic susceptibility test was performed by disc diffusion method. Based on the results *Lactobacillus paracasei* KUMBB005 showed resistant to Kanamycin, Erythromycin, Vancomycin and Streptomycin (Fig. 7). These results concur with [22] who reported that *Lactobacillus* reveal resistance to various classes of antibiotics such as β lactam ring and aminoglycoside group. The resistant property of *Lactobacillus* against some antibiotics would be associated with preventive and therapeutic purpose in controlling intestinal infection. The antibiotic resistant quality of *Lactobacillus paracasei* is the major concern because of the efficacious.

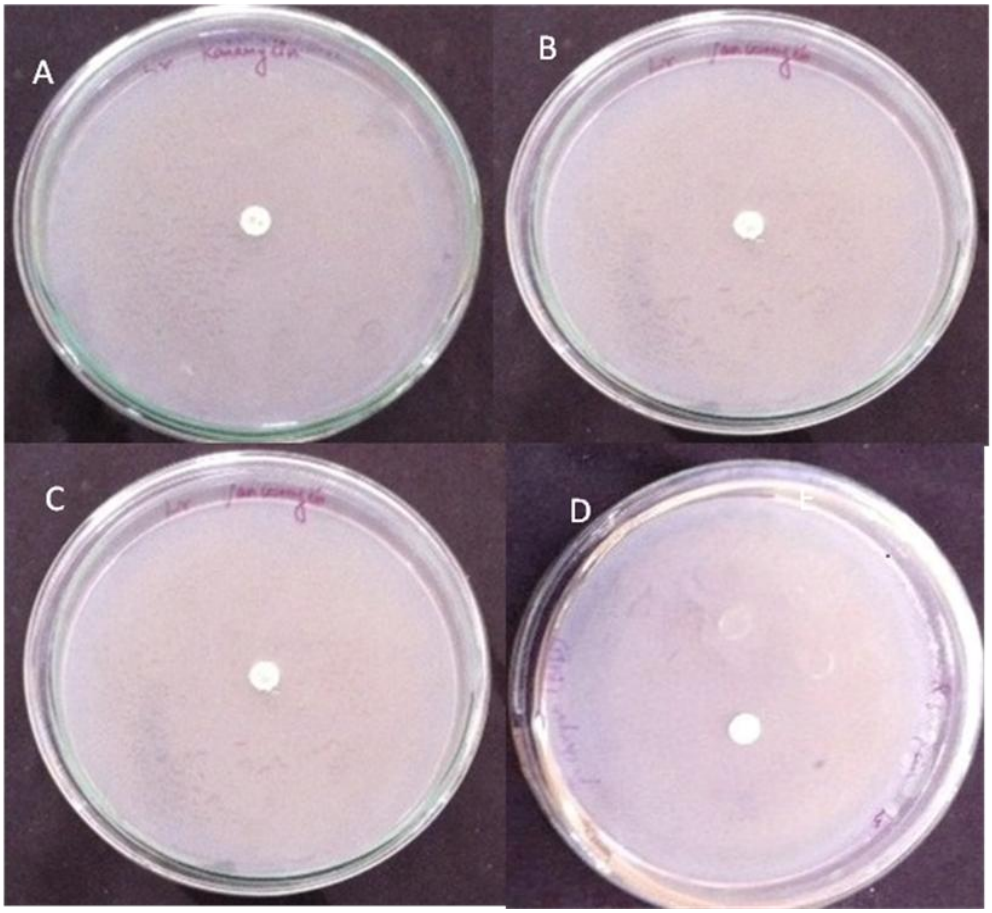

Fig. 7. Showing antibiotic susceptibility of *Lactobacillus paracasei* KUMBB005

A- Kanamycin, B- Erythromycin, C- Vancomycin, D- Streptomycin

CONCLUSION

In conclusion, various *Lactobacillus* isolates (KUMBB001, KUMBB002, KUMBB003 and KUMBB005) do exist in the fresh cow milk samples, the isolates be exploited as a probiotic after investigating its beneficial characteristics. The isolate KUMBB005 fulfills the required

character for a *Lactobacillus* sp., such as tolerance to conditions such as acidic pH 6, 1-9% NaCl concentration, 1-5% bile (Ox-gall) salt concentration and produce extracellular antibacterial substance that inhibits pathogenic test organisms. The isolate KUMBB005 was resistant to various test antibiotics. Further the identification through 16S rRNA sequence reveals the isolate KUMBB005 as *Lactobacillus paracasei*. Therefore from this study it is considered that this isolate can be potential use as probiotic organism for future research process.

ACKNOWLEDGEMENT

The authors are sincerely grateful to the management authorities at Karpagam University, Coimbatore – 641 021, for their constant encouragement and support.

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