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Molecular characterization of *lactobacillus sp,* from Indian curd and its Antagonistic effects on uropathogens of Diabetic patients

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Abstract

Diabetes mellitus is a metabolic disease and is associated with increased risk of certain complications with UTIs. The LABisfamous to yield antibacterial substances likebacteriocins which can inhibit the growth of a number of UTI pathogens. The fermented probiotic food (curd) samples were collected from home and supermarket in Tamil Nadu, India. Meanwhile the urine samples were collected from clinically UTI suspected diabetic patients from private hospitals in Tamil Nadu, India. The foremost aim of this study is to identify the different Lactobacillus sp., from curd and uropathogens from UTI diabetic patients by 16S rRNA gene sequencing. This study also stretched to extraction of bacteriocin from identified Lactobacillus sp., by fermentation and the study overextended to antibacterial activities of bacteriocin against on an identified uropathogens. All the isolates were identified by 16S rRNA sequencing. Antimicrobial activities of LAB bacteriocin was done against on UTI pathogens. L.acidophilus JCM1132, L. fermentam BCS25, L. plantarum ATCC8041 and L. casei XM2-1 from curd and Streptococcus sp, Staphylococcus sp, E.coli sp, Bacillus subtilis sp, and Pseudomonas sp were identified by 16S rRNA sequence. The fermentation process was optimized for getting maximum of products that gave antimicrobial activity against UTI pathogens. In this study, bacteriocins of L.acidophilus JCM1132 and L.fermentam BCS25 as a potential broad spectrum antibiotic against on an uropathogens (Streptococcus sp, Staphylococcus sp, E.coli sp, Bacillus subtilis sp, and Pseudomonas sp), but L.plantarum ATCC8041 and L. casei XM2-1 are mild antimicrobial activities on few isolates.

Keywords: Lactic acid bacteria, antimicrobial activity, bacteriocin-like compound, probiotics, urinary tract infection, fermentation.

Introduction

The curd is an Indian traditional fermented milk product that used as a main food sources in our routine life. The fermented milk products are probiotics that contains viable lactic acid bacteria (LAB) and its metabolic by-products act as an antioxidant, immune modulator and antimicrobial agents. LAB is a group of Gram-positive bacteria united by a constellation of morphological, metabolic, physiological characteristics[1]. It is indigenous inhabitants of the human gastrointestinal tract, and is thought to be among the dominant colonists of the small intestine[2]. LAB exerts a strong antagonistic activity against many human pathogens as a result of the production of organic acid, hydrogen peroxide (H_2O_2), inhibitory enzymes and bacteriocin[3-5]. Many of these lactic acid bacteria are known to produce antibacterial substances including bacteriocins which can inhibit the growth of several pathogenic bacteria[6]. The increase in bacterial resistance to various antibiotics has stimulated investigators around the world to improve disease control strategies, which has led to the discovery of new vaccines and non-specific immune-stimulants. Thus, there is a growing interest worldwide in the use of probiotic bacteria for their various beneficial influences on animal and human health[7]. The bacteriocins from LAB isolated from other traditional foods of India have proven its applicability in biopreservation[8,9]. Bacteriocin is known to be altered in different environmental conditions and optimum production may require a specific combination of environmental parameters. The production of bacteriocinis often regulated by microbial growth rate, substrates, pH and temperature[10]. The direct effects of probiotics in the GI tract are well documented and include up regulationof immunoglobulins such as IgA, down regulation of inflammatory cytokines, and enhancement of gut barrier function. The research evidence supports indirect, systemic effects of probiotics for a widely divergent set of disorders, including atopic disease, immune compromise and vaginal infections[11]. Diabetes mellitus (DM) patients have a high prevalence of asymptomatic bacteriuria (ASB) and urinary tract infections (UTIs). Diabetes has long term effects on the incidence of UTIs and has been reported to be around three to four times high in diabetic compared with non-diabetic patients[12]. The higher prevalence of ASB and higher incidence of symptomatic UTIs in diabetic patients, when compared to non-diabetic patients that often leads to UTI complications[13]. The aim of this study is to identify the different *Lactobacillus sp.,* from fermented milk products and uropathogens from urinary tract infected diabetic patients by 16S rRNA gene sequencing. This study also stretched to extraction of bacteriocin from identified

Lactobacillus sp., by desk top fermentation processes and the study overextended to antibacterial activities of bacteriocin against on identified uropathogens.

Material and Methods

Samples collection: There are two different types of samples were collected for an invitro association study. First, fermented probiotic 15 food (curd) samples were collected from different places (home and supermarket) of Tamil Nadu, in India. Meanwhile the second sampling enrolled, a totally 10ml of 30 midstream urine samples were collected in screw capped sterile container from clinically UTI suspected diabetic patients in private hospital in Tamil Nadu, India. Both samples were collected under sterile manner and stored at -70°C for future evaluation of microbiological and biochemical characteristics.

Microbiological screening of bacteria from two different samples: The stored samples were brought out to room temperature from -70°C and opened under sterile manner in laminar airflow hood. One gram of solid fermented curd was weighted and transferred aseptically into physiological saline (0.9% NaCl) and homogenate all the samples in one sterile flask. Serial diluted 1 ml of samples was pour plated on MRS agar (Hi-media, India). All plates were incubated at 37°C for 24-48h under microaerobbic conditions until visible colonies appeared and then selected colonies inoculated into De Man, Rogosa and Sharpe (MRS) broth without aeration. The isolates were preserved in 15% (v/v) glycerol containing MRS broth at-70°C till future processing. After that, from 10ml of all mid-stream urine samples, directly 1ml of samples were inoculated on nutrient agar (NA), McConkey agar (MC), triple sugar iron agar (TSI), Eosin methylene blue agar (EMB), Hi-crome UTI agar, Baird-Parker agar, Cetrimide agar and blood agar (BA) were purchased from Hi-media, India, and incubated at 37°C for 24-48 hrs. The isolates were identified by colony colour, morphology and biochemical tests. The Gram staining and biochemical analysis of cultured isolates were examined by standard methods. The selected colonies based on the cultural, microscopic and microbiological examinations, were subjected to biochemical examination (starch hydrolysis, lipid hydrolysis, casein hydrolysis, triple sugar iron agar test, oxidase test, catalase test, nitrate reduction test, indole production test, methyl red test, voges-proskauer test, citrate utilization test, urease test**)** for confirmation of the pathogens. The selected colonies were inoculated in MRS/nutrient broth for making pure culture and store at -70°C for further use.

Genomic DNA extracted from various isolates: Pure cultures were obtained by sub culturing through number of times from the original cultures. The cultures were grown on Luria-Bertani (LB) broth and incubated at 37°C for 48-72 hrs for DNA isolation. The bacterial genomic DNA was extracted from 200µl of suspension culture cells (104 -108) prepared from pure culture of various isolates by using phenol-chloroform DNA extraction method. The DNA pellet was dissolved in 200µl of TE buffer and stored at 4°C for further use. The extracted DNA was qualitatively checked by electrophoresis with 0.8% agarose gel and observed the band under UV trans- illuminator and then purity and quantity tested by using the cuvette free nanodrop spectrophotometer (ND-1000).

Molecular identification of isolates by using 16S rDNA sequencing: The selected isolates were examined microscopically to identify thecolonies and cellular morphology and Gram staining phenotype. The 16S rRNA gene LAB strains were amplified by using polymerase chain reaction (PCR) technique. The 16S rDNA gene was amplified by PCR with a thermal cycler (SureCycler 8800, Agilent technologies, USA). DNA fragments of approximately 1.5 kpb were amplified using the primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGYTACCTTGTTACGACTT-3). Each PCR tube (50µl) contained a reaction mix of 10µl 5XPCR buffer for Taqpolymerase (Promega), 1.5mM MgCl2, 200 µM of each deoxynucleotide triphosphate (Promega), 0.4 µMof each primer and 2Uof TaqPolymerase (Promega) and 5µl of template DNA. PCR temperature and cycling profiles included a hot start at 96°C /5 min, 25 cycles consisting of denaturation at 96°C /1 min, annealing at 50°C /1 min, extension at 72°C /2 min, and a final extension at 72°C (10 min.). PCR products were determined by electrophoresis in 1% (w/v) agarose gel and visualized by ethidium bromide (1µl/10 ml) staining. PCR Clean-Up Kit (CHROMOUS BIOTECH, Bangalore, India) used for rapid purification of single-stranded or double-stranded PCR amplification products (100bp to 10kb) from the other components in the reaction such as excess primers, nucleotides, DNA polymerase, oil, and salts. The purified PCR products of approximately 1,400 bp were sent to CHROMOUS BIOTECH, Bangalore, India for sequencing, by used 2 primers 518 F (5'-CCA GCA GCCGCG GTA ATA Cg -3') and 800R (5'- TAC CAG GGTATCTAATCC -3'). Where the both strands of the purified PCR products were sequenced by an ABI 3100 automated gene sequencer (ABI, Forster, USA).

Phylogenetic analysis: The phylogenetic analysis was comprehended by an alignment of sequence consensus of the 16S rDNA genes together in an international database (Genebank). The sequences were compared with the sequences deposited in the GenBank database using the BLAST algorithm. i. These partial sequences were searched against sequenced deposited in GenBank using the advanced BLAST similarity search option. ii. The 16S rRNA gene sequences that had a greater than 98% similarity to sequences deposited in GenBank were designated as belonging to the corresponding species.The Molecular Evolutionary Genetics Analysis 6 (MEGA6) software was used for constructing the phylogenetic tree for identified bacteria from two sources. Divergence times for all branching points in the user-supplied topology were calculated using the Maximum Likelihood method based on the General Time Reversible model.

Optimization of bacteriocin fermentation by lactobacillus sp: The optimization was performed with different concentration energy sources and various parameters. Optimization leads to maximize the growth of organism and to increase the yield of the product. This optimization implicates different conditions like carbon source, nitrogen source, temperature, and pH and these should be affecting the metabolic by-products of microorganisms. Refined and pure carbohydrates of Carbon source like sucrose, glucose, fructose, dextrose were used in the lab scale to optimize the growth and yield of product. The nitrogen sources of inorganic or organic sources supply the nitrogen energy to the microbial fermentation. Mainly yeast and beef extracts have high content of nitrogen source that leads to more growth of microbes than other sources. The effect of temperature on fermentation was carried out at 4°C, 25°C, 37°C, and 42°C for production of bacteriocin by using *lactobacillus* strains. For the successful bacteriocin fermentation, maintenance of favourable pH is crucial. Hence the effects of initial pH (5.0, 6.0, 7.0, and 8.0) on the growth of *lactobacillus sp.,* strains and its bacteriocin production were studied. Mineral acids such as HCl or sulphuric acid and NaOH are used to adjust the pH of the fermentation media. The optimized parameters were analyzed by Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA) enables highly accurate of 1ul samples with remarkable reproducibility. The fermentation process was done followed by above optimized conditions and parameters.

Upstream fermentation process: Microorganism can be grown in culture tubes, flasks, and stirred fermenters or other mass culture systems. This is most critical during scale-up, where a successful procedure developed in a small flask is modified for use in large fermenter. The inoculum (lactobacillus sp.) were prepared in usual nutrient broth containing 250 ml flask incubate at 37°C for 48 hours to starting small scale-up fermentation for the production of the bacteriocin, a complex medium is formulated from the case studiesbacteriocin of the production. Bacteriocins were produced by submerged fermentation process that was performed by the optimized conditions of temperature, pH, and carbon and nitrogen source. The following chemical composition used for making fermentation medium: Tryptone (30g), Yeast extract, (30g), Beef extract (30g), Tri ammonium citrate (2g), Sodium acetate (5g), Manganese sulfate (1g), Dipotassium hydrogen phosphate (2g), Sodium chloride (30g), Glucose (30g), Tween 80 (10ml) that all were dissolved in 1000 ml of Distilled water. One liter of production medium is prepared with above composition and Autoclave medium at 121^0C for 6 hrs. The medium allowed to room temperature for two hours and add the 50ml of prepared inoculum into fermenter. Allow the fermenter to run for 48 hours as per optimized (temperature at 37^0C, pH 7-8, and rotation at 400rpm/min) and other typical parameters. Bacterial growth was determined by measuring Optical density (OD) at 580 nm using a NanoDrop 1000 spectrophotometer.

Downstream fermentation processing: Bacteriocin is an extracellular product of *lactobacillus sp*., fermented crude upstream product that can be extracted and purified by three major methods followed such as i. centrifugation ii.filtration and iii. Thin Layer Chromatography. The product of upstream fermentation contains whole cells, media, and secondary metabolites that separated based on their size and mass by ultracentrifugation. The bacteriocin is a secondary metabolite, which was present in supernatant that were separated by centrifugation at 30000 rpm/m for 20 minutes. Ultrafiltration is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained, while water and low molecular weight solutes pass through the membrane. This separation process is used in industries and research for purifying and concentrating macromolecular solutions, especially protein solutions. The centrifuged supernatant was allowed to ultrafiltration at 4°C for 15 minutes (0.22µm, Millex-GV filter, Millipore) for isolate macromolecule from water, electrolytes containing supernatant. Thin layer chromatography is a method for identifying substances and testing the purity of compounds. TLC is a useful technique because it is relatively quick and requires small quantities of material. Silica gel as mobile phase was prepared and poured into glass slide and kept in hot air oven for 40 minutes. The filtrate with solvent was placed using capillary tube at the end of slide. The slide was placed in the solvent prepared beaker that contains butanol: methanol: 16% aqueous ammonia in the ratio of 5:4:3(v/v). Cover the solvent containing beaker and it was allowed to rise until it almost reaches the top of plate that will gave the maximum of the dye compounds for this particular combination of mobile and stationary phase. The plates were run for 10minutes and the chromatogram was air dried and 0.1% of ninhydrin solution was sprayed and fluorescence was observed under UV trans-illuminator at 245nm.

Anti-uropathogenic activities of Lactobacillus sp.: The separation of bacteriocin from downstream processing it is meant to assay by preparing the Muller-Hinton (M-H) agar medium. M-H agar medium were Prepare and Autoclaved the medium at 121^0C for 15 minutes. Add 1ml of isolates (*E.coli, Pseudomonas sp, staphylococcus sp, streptococcus sp, and Bacillus sp*) in the corresponding MH agar medium. After solidified the agar, Wells made by well puncher on the agar plates and then 100µl of suspended bacteriocin extracts (*L. casei XM2-1, L.acidophilus JCM1132, L.fermentam BCS25, andL.plantarum ATCC8041*) were added into the corresponding well in agar plate and incubated 24hrs at 37^0C.

Results and Discussion

Molecular identification of isolates by using 16S rDNA gene: All strains were isolated and identified by microbiological and biochemical techniques, initially. A total of 16 bacterial strains isolated from fermented dairy products and dairy fermentation were considered as belonging to the genus *Lactobacillus* based

on their morphology, Gram-positive staining, absence of catalase activity and ability to produce acid during growth. Whereas the UTI pathogens were identified based on colony colour, morphology and biochemical tests and totally 23 bacterial strains were carried out to confirm the presence of the species of uropathogens (like *Streptococcus, staphylococcus, E.coli, Bacillus subtilis and P. aeruginosa, Bacillus*). Hence to identify and confirm the desired isolates at molecular level, 16S rRNA gene region was amplified and sequenced. Genomic DNA was extracted from all the selected isolates by the phenol chloroform method. PCR amplification of 16S rRNA gene region by using universal primer, the obtained PCR product resulted in 1451 bp. By using BLAST analysis, out of 39 isolates, totally 9 isolates sequences of NCBI data gave 100% similarity. Four are lactobacillus strains (*L. casei XM2-1, L.acidophilus JCM1132, L.fermentam BCS25, andL.plantarum ATCC8041*) from fermented dairy products and remaining five are *Streptococcus sp. VAMC 3076, staphylococcus aureus CLRSA3, E.coli KVP104, Bacillus subtilis UDG CBCC2 and P. aeruginosa AU04*. Phylogenetic tree generated by The Molecular Evolutionary Genetics Analysis (MEGA) software tool proves that this organism genetically related within the isolated organisms. The interrelationships among identified strains of two samples of phylogenetic tree are shown on figure-1.

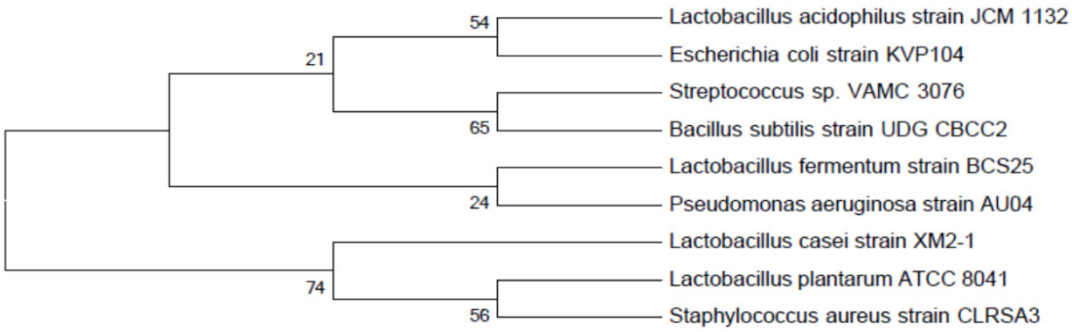


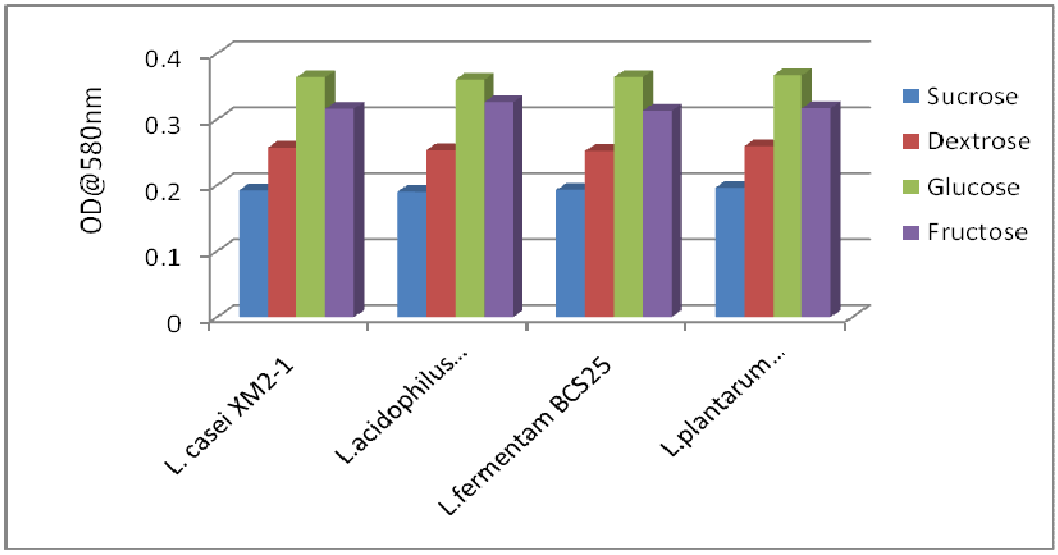
Figure-1
Molecular phylogenetic tree constructed using 16S ribosomal RNA gene sequences

Table-1
High levels of bacteriocin production ofoptimized energy sources and parameters for different *Lactobacillus sp*., at O.D at 580nm

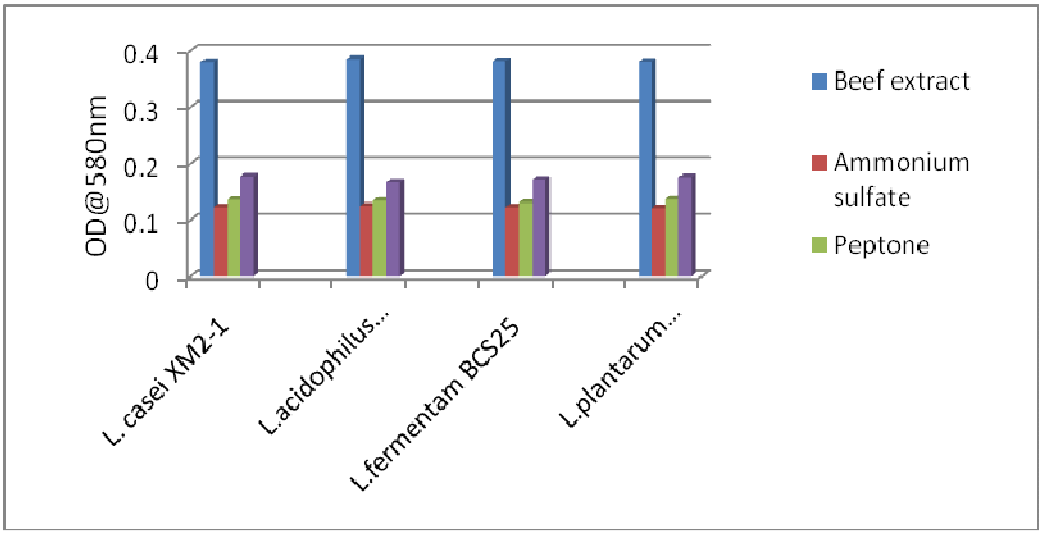
Sources/ parameter	Individuals	L. casei XM2-1	L.acidophilus JCM1132	L.fermentam BCS25	L.plantarum ATCC8041
Carbon source	Sucrose	0.192	0.190	0.193	0.196
	Dextrose	0.257	0.253	0.252	0.259
	Glucose	0.364	0.360	0.364	0.367
	Fructose	0.316	0.326	0.313	0.317
Nitrogen source	Beef extract	0.380	0.385	0.382	0.381
	Ammonium sulfate	0.123	0.125	0.123	0.121
	Peptone	0.136	0.134	0.131	0.137
	Urea	0.177	0.167	0.172	0.176
Temperature	4^0C	0.126	0.128	0.129	0.122
	25^0C	0.300	0.310	0.307	0.298
	37^0C	0.838	0.818	0.832	0.817
	42^0C	0.692	0.690	0.682	0.679
pH	5.0	0.043	0.049	0.050	0.049
	6.0	0.087	0.101	0.098	0.110
	7.0	0.465	0.455	0.461	0.471
	8.0	0.339	0.331	0.329	0.333
Log time	8 hours	0.091	0.089	0.092	0.100
	16hours	0.201	0.199	0.210	0.212
	24hours	0.329	0.321	0.331	0.340
	32hours	0.498	0.501	0.538	0.582
	40hours	0.502	0.508	0.509	0.510
	48hours	0.199	0.220	0.211	0.218

Optimization and production of bacteriocin: Above four lactobacillus strains are100% similarity of existing organisms and brought out from stored corresponding pure culture. Optimization was done against various degree of parameter and different concentration of energy sources. These preliminary optimal conditions for bacteriocin production were subsequently combined with an alteration in the initial carbon source of sucrose, dextrose, glucose, fructose concentrations of 30 g per litter. Maximal bioactivity (0.164 OD @580nm) was obtained with 30g glucose per litre. Likewise, optimized submerge fermentation results were showed that 30g/l of glucose, 30g/l of beef extract, pH: 8 and temperature at 37°C for maximal bioactivities / production of bacteriocin. All optimized parameters and energy sources are given in table-1 and that helps to improve maximum level of bacteriocin production and avoid microbial contamination. The growth curve shown about maximum level of bacteriocin in figure-2.

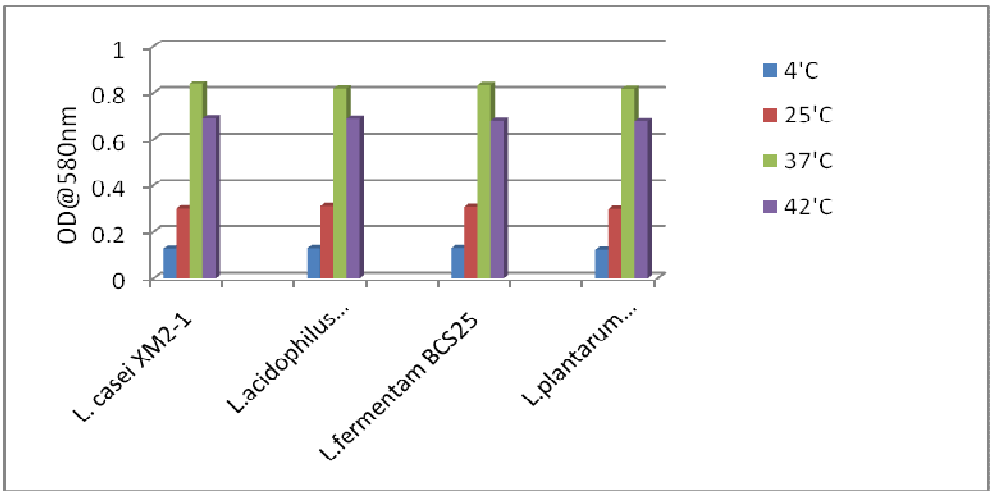
Antimicrobial activities of bacteriocins: The results were observed and measured the diameter of zone formation of antimicrobial (*L. casei XM2-1, L.acidophilus JCM1132, L.fermentam BCS25, and L.plantarum ATCC8041*) activities against on M-H agar plates of*Bacillus subtilis, E. coli, Staphycoccus, Streptococcus and Pseudomonas.* The *L.acidophilus JCM1132* and *L. fermentam BCS25* gave more antimicrobial activities above uropathogens, but *L.acidophilus JCM1132* were less in *Pseudomonas sp.* Whereas *L.plantarum ATCC8041* gave moderate antimicrobial activities to *Bacillus subtilis, Staphycoccusand Pseudomonas,* but there was no effects on, *E. coli,* and, *Streptococcus.* There was no antimicrobial activies of *L. casei XM2-1*against to above mentioned uropathogens, but it furnished false zone on *Staphycoccus.* The detailed diameters of bacteriocinzone against to uropathogens are listed in table-2 and M-H plateshown on figure-3.



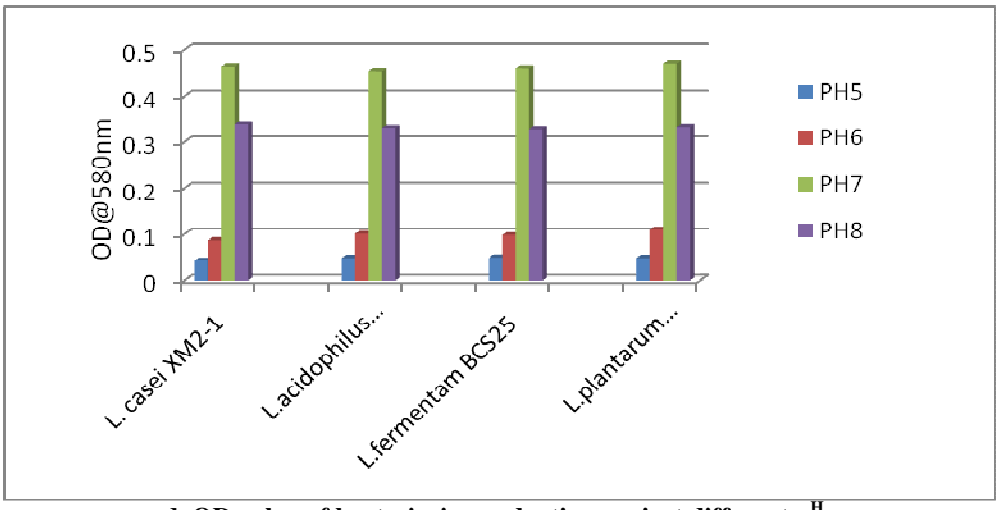
a. OD value of bacteriocin production against various carbon sources:



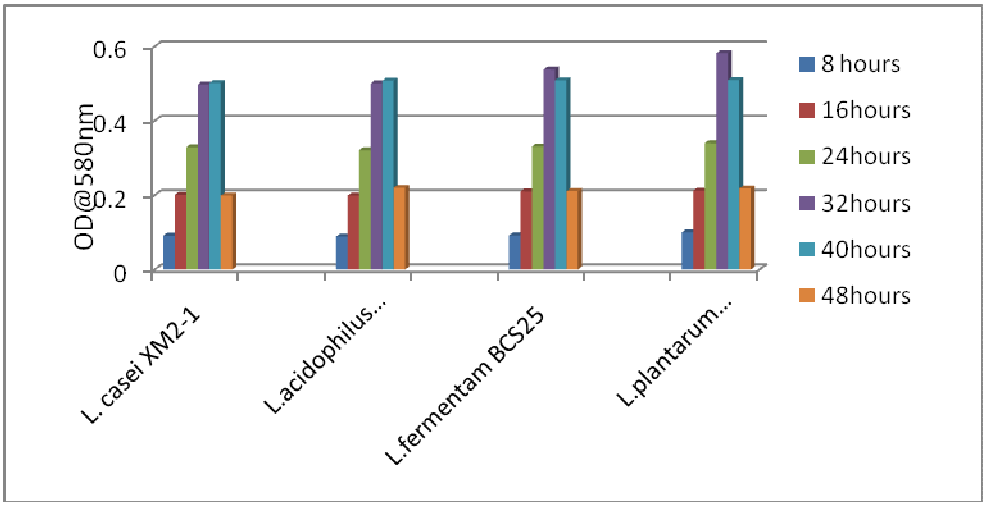
b. OD value of bacteriocin production against various nitrogen sources:



c. OD value of bacteriocin production against different temperature:



d. OD value of bacteriocin production against different p^H:



e. OD value of bacteriocin production against different logarithmic time:

Figure-2

Parametric optimization and growth curve studies of bacteriocin production are shown on a, b, c, d, and e against OD @ 580nm.

Discussion: The conventional methods for the identification and Characterization of clinical isolates of bacterial pathogens by DNA sequencing technology have greatly enhanced the ability of the microbiologist to determine the identity of a bacterial isolate[14]. The 16S-23S intergenic spacer region has been used to characterize certain pathogenic agents and compare these to homologous region within *E.coli*. Phylogenetic analyses inferred from this molecular target were similar to those obtained by analyses derived from 16s rDNA and Housekeeping genes[15]. The antimicrobial active compounds like lactic acid and reduction of pH, and H2O2, acetic acid, fatty acids, aldehydes and other compounds produced by LABs[16]. There was no any correlation between H2O2, lactic acid and bacteriocin activity, and also found that three LAB strains produced H2O2, but did not had any validated inhibitory effect[17]. Another similar study reported that *L.lactissubsp* strain produced high level of lactic acid but there was no H2O2 production. Moreover, this strain did not had inhibitory effect against *E.coli* and *P.aeroginosa*, but had inhibitory effect of on *S.aureus*[18]. Xanthopoulos et al. reported that *L. acidophilus* and *L. paracasei subsp. Paracasei* strains isolated from infant faces, which had weak antibacterial activity on *E. coli* and *Y.enterocolitica*[19]. Diabetic patients with UTIs are mostly considered as complicated UTIs, so that clinical experts recommended the long-term treatment for Diabetic patients with UTIs than UTIs without diabetic patients[13]. In 1994, the World Health Organization (WHO) deemed probiotics to be the next-most important immune defence system when commonly prescribed antibiotics are rendered useless by antibiotic resistance[20]. Serum cholesterol concentration reduced after consumption of yogurt enriched with a specific strain of *L. acidophilus*[21]. The colon might thus be a source of beneficial as well as harmful bacteria for the urinary and genital tracts. The controlled clinical studies are needed to substantiate these preliminary findings. Both oral probiotics and vaginal suppositories of probiotics have been shown to reduce the incidence of recurrent urinary tract infection[22]. The critical factors in any successful application of probiotics to patient care are the scientific basis for selecting probiotic strains and clinical verification that they are effective against the recurrence of UTI. Three strains like *L. rhamnosus* GR-1 and *L. fermentum* B-54 and RC-14—have been shown to colonize the vagina and act as a barrier to the ascension of uropathogens into the bladder[23].

Table-2

Antibacterial activities of four different strains of *Lactobacillus sp*, against on *Streptococcus sp, Staphylococcus sp, E.coli sp, Bacillus subtilis sp, and Pseudomonas sp*, by means of M.H agar plates

Serial No.	Urinary tract infected bacteria	Effects of bacteriocin radius in diameter (cm)			
		L. casei XM2-1	*L.acidophilus JCM1132*	*L.fermentam BCS25*	*L.plantarum ATCC8041*
1.	*Streptococcus sp,*	0	1.1	**2.2**	0
	Staphylococcus sp,	0.1	0.9	2.1	0.2
	E.coli sp,	0	1.2	1.9	0
	Bacillus subtilis sp,	0	0.5	2.1	0.3
	Pseudomonas sp,	0	0.9	0.2	0.2

= highlighted numerical are more antimicrobial activities**.**

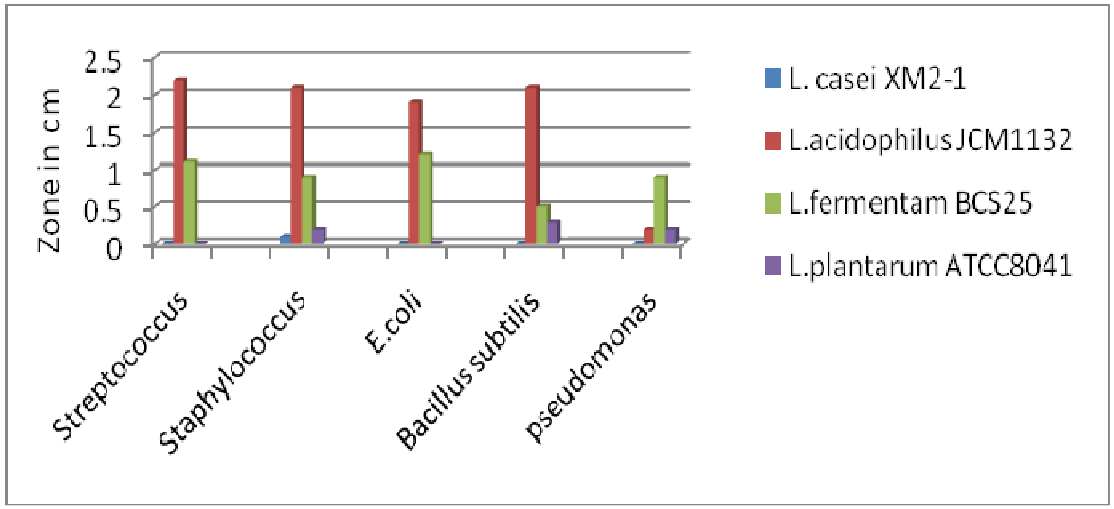


Figure-3

The graphical representations of antimicrobial activities *Lactobacillus sp*., against five uropathogens were assessed using the agar well diffusion method

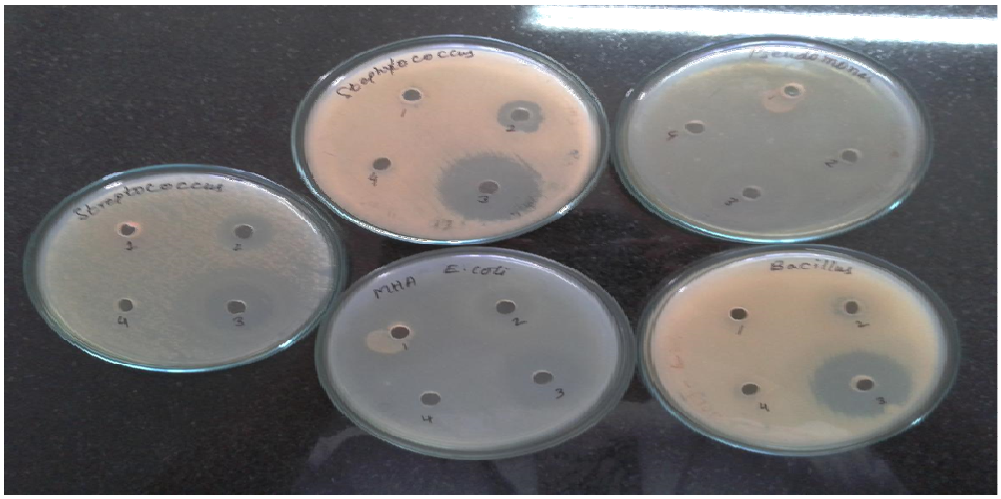


Figure-4
Antimicrobial activities assay result of *lactobacillus sp*., against on isolated uropathogens

Conclusion

The role of LAB may involve in modifying gut pH, antimicrobial activities, immunomodulation, and control of serum cholesterols and cancer. LAB producing bacteriocin acts as an antimicrobial substance that is an antagonist to some UTI pathogens and it may also modulate / stimulate immune response. In this study, bacteriocins of *L.acidophilus*JCM1132 and *L.fermentam* BCS25 as a potential broad spectrum antibiotic against uropathogens (*Streptococcus sp, Staphylococcus sp, E.coli sp, Bacillus subtilis sp, and Pseudomonas sp)*, but *L.plantarum* ATCC8041 and *L. casei* XM2-1 are mild, false positive antimicrobial activities respectively. Still probiotic bacteriocin stimulated immune response is unknown, so this study needed to carried out to understanding of LAB stimulated immune response in in vivo trails. This study also needed to be conformed the large numbers of pathogenic bacteria from UTI against various LAB producing antibiotics.

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