

Original Research Article

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Antimicrobials from Wild Strains of Lactic Acid Bacteria for Commercial Applications

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ABSTRACT

This study aims to screen wild strains of lactic acid bacteria for antimicrobial activity to control the food borne pathogens as natural biopreservatives to reduce the ill effects of synthetic preservatives. The antimicrobial efficacy of 14 Lactic acid bacteria (LAB) isolated from fermented food samples were tested. Nearly 19 food borne pathogens were isolated from various spoiled food samples and were used as test organisms. The LAB cultures were characterized by Gram staining, catalase test, carbohydrate fermentation test, temperature tolerance, pH tolerance, antibiotic sensitivity and lysozyme tolerance. Biochemical analysis like exopolysaccharides production, titrable acidity, MR-VP and esculin hydrolysis were also done. LAB isolates were tested for their antimicrobial activity against 19 food borne pathogenic test organisms. Out of 14 isolates, only 5 LAB cultures exhibited antimicrobial activity by forming inhibition zone against pathogens. The zone of inhibition was recorded maximum for CS (1.33cm²) and CT (1.13cm²) isolates. The LAB strains showing maximum antibacterial activity were identified as *Streptococcus thermophilus* and *Lactobacillus coryniformis* respectively by 16S rRNA sequencing. This study was resulted in identification of LAB isolates from natural fermented foods for antimicrobial activity. The antimicrobial compounds may be of future interest which can be characterized and applied for biopreservation of foods.

Keywords

Antimicrobials,
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Introduction

Food safety and food hygiene is the most important concern towards the public health caused by food contamination and food-borne diseases. In addition, ill-effects of chemical preservatives, the demand for natural food preservatives has reached its peak in recent

years (Gyawali and Ibrahim, 2014). Lactic acid bacteria (LAB) have the ability to ferment food and possess higher nutritional values. Fermentation of foods enhance taste, flavour and texture of food and also exert antimicrobial activity by inhibiting the proliferation of pathogenic and spoilage microorganisms. Thus LAB can be used as a

natural food preservative (Indira *et al.*, 2011). The antimicrobial activity exerted by the LAB is due to the production of active metabolites like organic acids, hydrogen peroxide, diacetyl, carbon di oxide, bacteriocins and bacteriocin like inhibitory substances (BLIS) which enhance the safety and extends the shelf life of food (Aymerich *et al.*, 2000; Favaro *et al.*, 2015). Hence this study aims at screening and isolating the LAB from 8 different fermented food sources and to select the best isolated culture which exhibits strong antimicrobial activity against food pathogens. Thus these strains or their formulations with best antimicrobial activity can be further used for commercial application as biopreservatives.

Materials and Methods

Isolation and selection of lab strains

Lactic Acid Bacteria were isolated from fermented food samples *viz.*, cumbu gruel, milk fermented by chilli, cold rice, neera, mango pickle, palmyrah fruit, fermented finger millet and sauerkraut. The samples were serially diluted (10^{-2} and 10^{-3}) and plated using spread plate technique in de Man Rogosa and Sharpe (MRS) agar media and incubated for 48 to 72 hours at $28 \pm 2^{\circ}$ C. Well isolated single colonies from each samples were further purified and preserved.

Gram reaction

Purified LAB isolates were examined for their morphological structure and Gram staining to confirm the identity of the isolated lactic acid bacterial cultures.

Catalase test

Lactic acid bacterial isolates were tested for the production of catalase enzyme using 3% of hydrogen peroxide. The cultures were

spotted over MRS agar plates and incubated for 24 h. Hydrogen peroxide was flooded over the spotted cultures on the plates. Catalase positive was indicated by the formation of gas bubbles over the culture and

Methyl red test

LAB cultures were inoculated in test tubes containing 5ml of MRVP broth and they were incubated for 48 h. To each tube 5 drops of methyl red indicator was added and observed for colour change. Formation of red colour indicates positive result and colour change from red to yellow indicates negative result.

Voges-Proskauer test

LAB cultures were inoculated in test tube containing 5ml of MRVP broth and they were incubated for 48 h. To each tube 12 drops of alpha naphthol solution and 2-3 drops of 40% potassium hydroxide were added. The tubes were kept under shaking for 30seconds and exposed to air for 15-30mins. Colour changes from crimson to ruby pink indicate positive result.

Sensitivity to Antibiotics

Antibiotic sensitivity of isolates were tested against amikacin (30 μ g), ciprofloxacin (5 μ g), gentamicin (10 μ g), ceftazidime (30 μ g), cefepime(30 μ g), ceftoxitin(30 μ g), cefoxatime(30 μ g) and ceftriaxone(30 μ g) by antibiotic disc assay. Formation of inhibition zone was observed and measured after 48h of incubation.

Carbohydrate fermentation test

Fermentative ability of lactic acid bacterial cultures for various carbohydrates like glucose, sucrose, lactose, mannitol and malic acid were tested. The culture tubes with fermentation broth were added with specific

carbohydrate at 1% level with inverted durham tube for detection of gas production. Phenol red was added as pH indicator which turns from red to yellow upon reduction in pH due to acid production.

Determination of titrable acidity

Acidity was measured by titrating 5 ml of 24 h old culture broth against 0.1N NaOH using phenolphthalein as indicator. Titration was done till the appearance of pink colour, and the titrable acidity in terms of lactic acid was determined using the formula given below (Sadler *et al.*, 2010; Adeyemo *et al.*, 2018).

$$\text{Titrable acidity\%} = \frac{V_1 \times N \times \text{Eq.wt} \times 100}{V_2 \times 1000}$$

Where,

V_1 = volume of titrant

N = Normality of titrant

Eq.wt = equivalent weight of acid

V_2 = volume of sample

Determination of pH tolerance

The LAB cultures were tested for acid tolerance by inoculating in MRS broth with different pH *viz.*, 1,3 and 5, adjusted using 0.1 N NaOH. Growth was measured after an incubation period of 72 h at 650nm.

Determination of temperature tolerance

LAB cultures were inoculated in MRS broth and incubated at temperatures *viz.*, 60⁰ C and 70⁰ C for 10mins and plated. Growth of cultures was measured after 72h.

Determination of exopolysaccharides production

Log cultures of isolates were centrifuged at 5000 rpm for 10 min and supernatant was

collected. In order to precipitate the EPS, 1ml of 90% ethanol was added to 0.5 ml supernatant and stored overnight at 4°C.

The mixture was centrifuged at 8000 rpm for 10min and the precipitates were suspended in distilled water and filtered. To 0.1 ml of filtrate 2ml of distilled water, 6% phenol and 5ml of 95% sulphuric acid were added. After 10 min absorbance was measured at 490nm (Chunlei *et al.*, 2014).

Determination of lysozyme tolerance

Agar well diffusion method was used to test the lysozyme tolerance of LAB isolates. LAB cultures were swabbed and wells were made on the culture swabbed agar plate. Lysozyme of concentration 1000ppm and 500 ppm was added to the wells and the culture growth was observed.

Esculin hydrolysis test

The bile esculin agar media was plated and the LAB cultures were spotted over the media and incubated for 48 h. Formation of dark blue colour indicates the positive result for hydrolysis of esculin.

Isolation of pathogenic test cultures

The standard pathogenic organisms *viz.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli* were obtained from department of Food Science and Nutrition, CSC and RI, Madurai. Also 16 more bacterial pathogens were isolated from potato(PTB), papaya(PYTB), apple (AOB), goat intestine(MSL,MPS and MWSB.), hen intestine (CWRB, CSG, CYB, CP, CSL and CSB) and fish wastes (FSB,FOB,FSL and FP) and characterised based on their morphology. LAB cultures were tested against totally 19 bacterial pathogens for antimicrobial activity.

Antimicrobial assay

Agar well diffusion method was used for testing the antimicrobial activity. Test pathogens were seeded into the Muller Hinton Agar (MHA) media and allowed to solidify. Using cork borer wells were made onto the seeded agar plate. Cell free supernatant 48h old LAB cultures were added to the wells. The plates were incubated at 37⁰ C for 48 h and the inhibition zone was measured.

Sequencing of 16S rRNA gene

LAB isolates found positive for antimicrobial activity against test pathogens were characterized by 16S rRNA gene sequencing using the universal primers 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT-3'). DNA was extracted from the selected two isolates and PCR amplification was done. The PCR products were custom sequenced by Eurofins Genomics India Pvt. Ltd., Bengaluru, India. BLAST tools available through the NIH (<http://www.ncbi.nlm.nih.gov/gov/blast/Blast.cgi>) were used to find out the similarity of DNA sequences (<http://www.ncbi.nlm.nih.gov/gov/blast>) and LAB culture was identified.

Results and Discussion

Isolation and purification

A total of 14 LAB cultures were isolated from the fermented food samples and characterized based on their morphology and biochemical tests. The isolated LAB cultures were named as cumbu gruel (CS and CT), milk fermented by chilli (CD), cold rice (CR), neera (NS and NT), mango pickle (MP), palmyrah fruit (PAL), fermented finger millet (FM(F)T,

FM(F)O, FM(FD)T and FM(FD)S) and sauerkraut (SKS and SKT) (Plate.1).

About 231 LAB strains were isolated from home fermented foods and studied for their antimicrobial activity (Ren *et al.*, 2018), from neera (Somashekaraiah *et al.*, 2019), from fermented fish peda (Putra *et al.*, 2018), from dairy products (El- Ghaish *et al.*, 2017) and from fresh fruits and vegetables (Linares Morales *et al.*, 2020).

Characterization of the isolates

All the cultures were found to be Gram positive and they were either rod and cocci shaped. In catalase test except 4 cultures *viz.*, FM(F)O, MP, SKS and NS, all the other isolates showed negative results by not producing any gas bubbles on addition of hydrogen peroxide. All the cultures except MP were positive lysozyme tolerance test and showed growth. All the cultures except NT and SKS showed growth at temperature 60°C and 70°C and thus were observed to be highly thermo tolerant (Table 1). Tolerance to acidity was tested at pH 1, 3 and 5. All the isolates were observed to be acid tolerant with maximum OD recorded as 0.496, 0.864 and 1.829 exhibited by NS, FM(F)T and FM(FD)S at pH 1,3 and 5 respectively (Table 4). Among the 14 LAB isolates 4 isolates *viz.*, FM(F)O, CD, FM(FD)T and FM(FD)S were sensitive to the tested antibiotics (Table 3; Plate.2). Indira *et al.*, (2011) has done the antibiotic resistance test for LAB isolates against 10 antibiotics. Li *et al.*, (2015) has demonstrated the temperature tolerance and acid tolerance of 59 LAB cultures isolated from corn stover silage producing antimicrobials and found the cultures were thermo tolerant and active at pH range of 2-6.

Table.1 Gram staining, biochemical characterization and temperature tolerance

LAB isolates	Gram Staining	Catalase	MRVP		Lysozyme Tolerance	Temperature Tolerance (10min)		Esculin hydrolysis
			MR	VP		60°C	70°C	
CS	+	-	+	-	+	+	+	-
CT	+	-	+	-	+	+	+	+
CD	+	-	+	-	+	+	+	+
CR	+	-	+	-	+	+	+	-
NS	+	+	+	-	+	+	+	-
NT	+	-	+	-	+	-	-	+
FM(F)T	+	-	+	-	+	+	+	-
FM(F)O	+	+	+	-	+	+	+	-
FM(FD)T	+	-	+	-	+	+	+	+
FM(FD)S	+	-	+	-	+	+	+	+
MP	+	+	+	-	-	+	+	-
PAL	+	-	+	-	+	+	+	-
SKS	+	+	+	-	+	-	-	-
SKT	+	-	+	-	+	+	+	+

Table.2 Carbohydrate fermentation test

LAB Isolates	Glucose		Sucrose		Lactose		Mannitol		Maleic acid	
	Gas	Growth	Gas	Growth	Gas	Growth	Gas	growth	Gas	Growth
CS	-	+	-	+	+	+	-	+	-	+
CT	-	+	-	+	+	+	+	+	-	+
CD	-	+	-	+	+	+	-	+	-	+
CR	-	+	-	+	+	+	-	+	-	+
NS	+	+	-	+	+	+	-	+	-	+
NT	-	+	-	+	+	+	-	+	-	+
FM(F)T	-	+	-	+	+	+	-	+	-	+
FM(F)O	-	+	+	+	+	+	-	+	-	+
FM(FD)T	-	+	-	+	+	+	-	+	-	+
FM(FD)S	+	+	+	+	+	+	-	+	-	+
MP	-	+	-	+	+	+	-	+	-	+
PAL	-	+	-	+	+	+	-	+	-	+
SKS	+	+	+	+	+	+	-	+	-	+
SKT	-	+	-	+	+	+	-	+	-	+

Table.3 Antibiotic sensitivity test

LAB cultures	Amikacin (30µg)	Ciprofloxacin (5µg)	Gentamicin (10µg)	Ceftazidime (30µg)	Cefepime (30µg)	Cefoxitin (30µg)	Cefoxatime (30µg)	Ceftriaxone (30µg)
CS	-	-	+	-	-	-	-	-
CT	-	-	-	-	-	-	-	-
CD	-	+	+	-	-	-	-	-
CR	-	-	-	-	-	-	-	-
NS	-	-	-	-	-	-	-	-
NT	-	-	-	-	-	-	-	-
FM(F)T	-	-	-	-	-	-	-	-
FM(F)O	-	-	-	-	-	-	-	-
FM(FD)T	-	-	-	-	-	-	-	-
FM(FD)T	-	-	+	-	-	-	-	-
FM(FD)S	-	+	+	-	-	-	-	-
MP	-	-	-	-	-	-	-	-
PAL	-	-	-	-	-	-	-	-
SKS	-	-	-	-	-	-	-	-
SKT	-	-	-	-	-	-	-	-

Table.4 Acid tolerance, exopolysaccharides production and titrable acidity

LAB cultures	pH tolerance OD value (650nm)			Exopolysaccharides OD value (490nm)	Titrable acidity (Lactic acid) (%)
	pH 1	pH 3	pH 5		
CS	0.021	0.087	0.147	0.02	0.576
CT	0.023	0.119	0.557	0.066	0.558
CD	0.007	0.065	0.636	0.088	0.414
CR	0.013	0.068	0.843	0.060	0.198
NS	0.496	0.325	1.133	0.010	0.126
NT	0.091	0.108	0.303	0.104	0.396
FM(F)T	0.055	0.864	0.656	0.052	0.54
FM(F)O	0.011	0.048	0.004	0.114	0.270
FM(FD)T	0.016	0.064	0.528	0.070	0.522
FM(FD)S	0.004	0.069	1.829	0.118	0.270
MP	0.007	0.709	0.635	0.020	0.216
PAL	0.006	0.051	0.625	0.121	0.324
SKS	0.008	0.037	1.720	0.109	0.180
SKT	0.045	0.071	0.068	0.064	0.432

Table.5 Antimicrobial assay

Lab culture	Pathogen	Area of inhibitory zone (cm ²)
CS	<i>Staphylococcus aureus</i>	0.126
	MSL	0.196
	<i>E.Coli</i>	0.196
	PYTB	0.332
	FSB	1.33
	FOB	0.112
CT	<i>Staphylococcus aureus</i>	0.096
	MSL	0.384
	<i>E.Coli</i>	0.126
	FSB	1.13
	FOB	0.10
CR	<i>Staphylococcus aureus</i>	0.283
	MWSB	0.096
	FOB	0.097
CD	<i>Staphylococcus aureus</i>	0.237
FM(FD) T	FSB	0.708

Plate.1 Purified LAB culture



Plate.2 carbohydrate fermentation and antibiotic sensitivity test

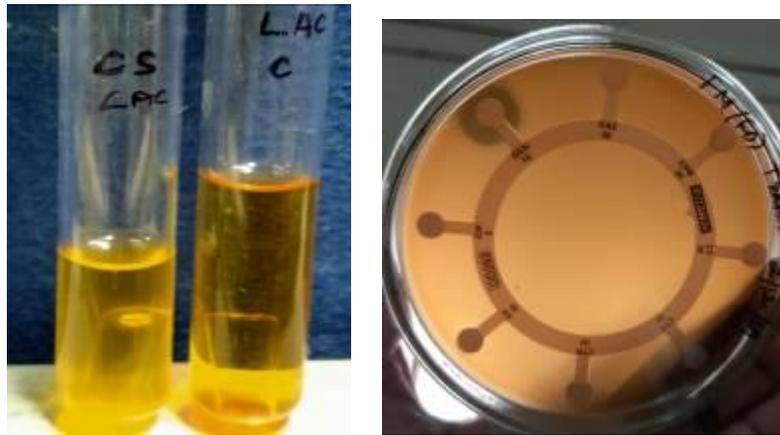


Plate.3 Antimicrobial assay of LAB isolates



Plate.4 Gel amplification of 16S rRNA gene

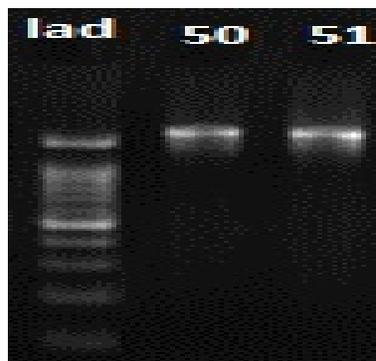


Plate.5 CT BLAST tree of *Lactobacillus coryniformis*

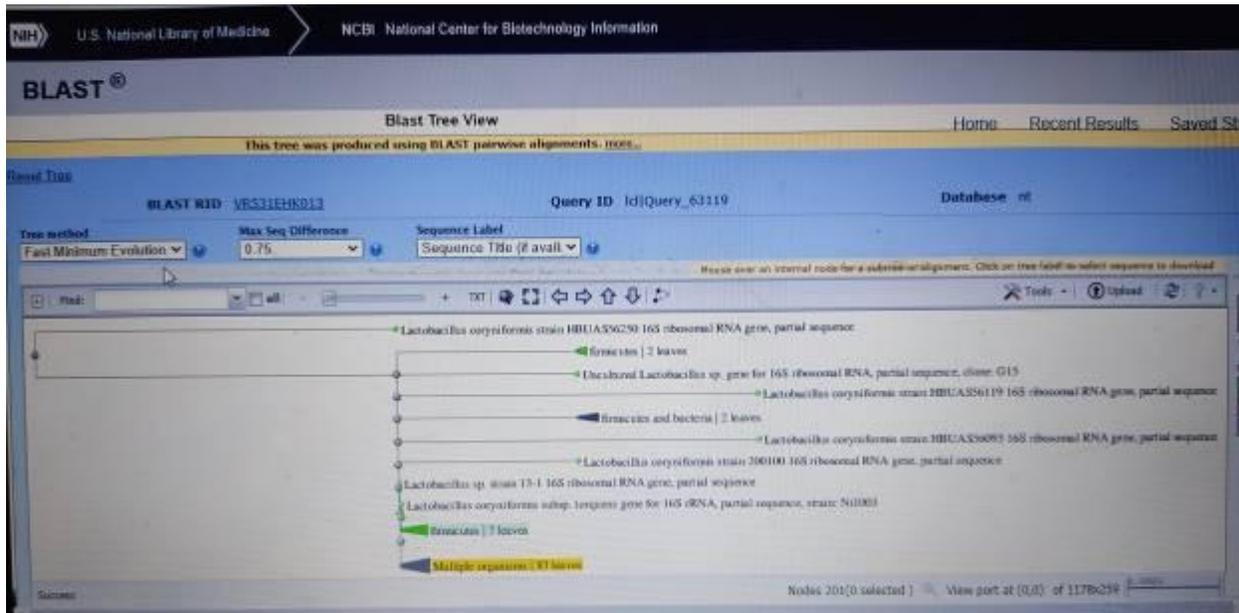
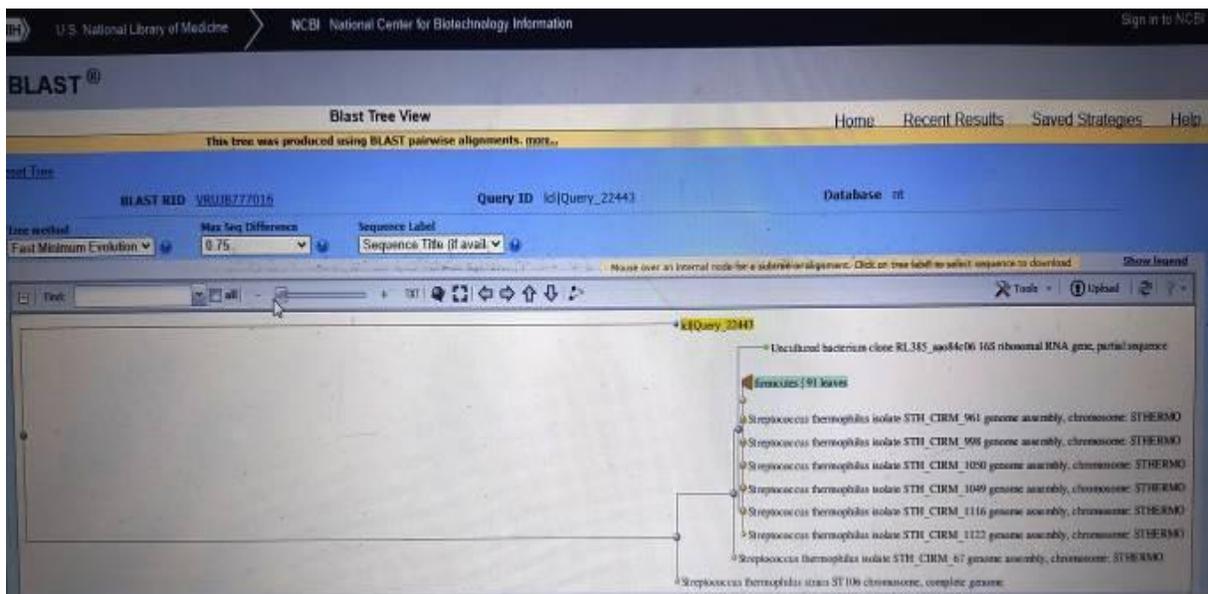


Plate.6 CS BLAST tree of *Streptococcus thermophilus*



Biochemical tests

The result of MR-VP test showed positive MR and negative VP reaction for all isolates.

The results of carbohydrates fermentation test is given in table 2 (Plate 2). All the cultures exhibited growth for all the tested sugars with varied gas production. Among the LAB

isolates titrable acidity was maximum in CS culture (0.576%) and minimum in NS isolate (0.126%). PAL culture recorded highest exopolysaccharide production (0.121) and NS culture recorded lowest exopolysaccharides production (0.010) (Table 4). Esculin was hydrolysed by 5 isolates viz., CT, CD, NT, FM(FD)T and FM(FD)S with dark blue colouration (Table 1). Kabuki *et al.*, (2007) tested *Streptococcus thermophilus* SBT1277 for esculin hydrolysi, and recorded the strain had not hydrolysed esculin. Chunlei *et al.*, (2014) has isolated 11 LAB cultures from inner mongolian traditional yoghurt and screened them for exopolysaccharide production and the highest range recorder is 539.9 mg/L.

Antimicrobial activity

Antimicrobial activity of 14 LAB cultures were investigated, where only 5 cultures viz., CS, CT, CR, CD and FM(FD)S exhibited antagonistic effect against 7 food pathogens tested out of 19 pathogens. CS and CT cultures exhibited the maximum inhibition zone of 1.33cm² and 1.13 cm² respectively against FSB (Table 5; Plate 3). Dung and Phong (2011) had studied 46 LAB cultures for their antimicrobial activity and concluded that 23 strains exhibited high antimicrobial activity where 7 of them showed strong effect with inhibition zone upto 10mm. Thu *et al.*, (2017) has tested 16 LAB cultures against *Bacillus subtilis* and all cultures possessed antimicrobial property.

16S rRNA sequencing

Among the isolated Lactic Acid Bacteria CS and CT exhibited strong antimicrobial activity against wide range of food borne pathogens. Hence the 2 cultures were identified based on 16SrRNA sequencing. BLAST was run which revealed the CS culture as *Streptococcus thermophiles* with 93.79% similarity and CT

culture as *Lactobacillus coryniformis* with 99.33% similarity (Plate.4; Plate.5; Plate.6)

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