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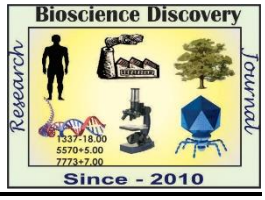

Isolation and characterization of lactic acid bacteria with probiotic potential from pickles

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Abstract

Pickle is a popular condiment throughout the world. In India different types of pickles are prepared from vegetables and fruits as side dish of vegetarian diet. Traditional methods of pickling in India involve lactic acid fermentation with addition of salt, spices, oil and jaggery. The objective of the research was to isolate predominant lactic acid bacteria (LAB) from pickles in West Bengal, India and study their antibacterial properties. The isolated LAB were differentiated phenotypically by morphological, biochemical and physiological characters. 16S rDNA sequencing confirms their identity as *Lactobacillus plantarum* and *Pediococcus acidilactici*. Antibiotic resistance pattern of the fourteen isolates against thirteen different antibiotics showed that most of the isolates were sensitive or intermediate against the antibiotics tested. All the tested isolates showed antibacterial activity by agar spot method against both gram positive (*Bacillus cereus*) and gram negative (*Escherichia coli* and *Salmonella* Typhimurium) bacteria. pH neutralized culture supernatant of one isolate from jackfruit showed antibacterial activity by agar diffusion method against all the three indicator organisms. This study concludes that LAB from pickles possess probiotic activity.

INTRODUCTION

Fermentation of substances leading to the production of wine, beer, bread, yogurt, cheese and pickles is regarded as the first biotechnological process devised and practiced by man from the time immemorial (Battcock and Ali-Azam, 1998). Indigenous fermented foods and beverages have been an integral component of the dietary culture of every community in the world ever since the beginning of human civilization (Tibor, 2007). Initially the primary objective of fermentation was to increase shelf-life of otherwise perishable raw materials. With the passage of time, fermented foods have become an integral part of our diet because of their multiple advantages. These foods can be prepared in the household or cottage industry with relatively simple techniques and equipments (Aidoo, 2006). Multidisciplinary systematic study involving microbiology, molecular biology, biochemistry and food technology has contributed immensely for large-scale industrialization of some traditional fermented products. Industrial production of fermented foods following scientific know-how has the advantage of maintaining consistent qualities, nutritional enrichment and expansion of the range of products over traditional household process as well as assuring microbial safety.

Fermented foods are made by the action of microorganisms, which may be indigenous to the raw material or added as starter cultures which modify the substrates biochemically and organoleptically into edible products (Tamang,

2007). India has a rich treasure of fermented foods produced from various ingredients like pulse, cereal, milk, fish, meat and vegetables. Diverse climatic condition, soil character and socio-cultural practices leading to different agricultural products and animal husbandry practices have resulted in hundreds of fermented products in India. Exploration of these lesser known fermented foods and characterization of predominant microorganisms may have an immense impact to the mankind because of their beneficial roles.

Pickling is one of the oldest methods of food preservation and has been practiced widely. They are made through the natural fermentation of fruits and vegetables. Besides adding nutritive value, pickles act as condiment and palatability enhancer (Joshi and Bhat, 2000; Savitri and Bhalla, 2007). During preparation of pickle, fully matured fresh vegetables and fruits are washed and cut into smaller pieces, salt added and generally sundried for 3-5 days. Various spices are added and the dried materials are then kept in closed jar filled with oil or jaggery. The pickles get ready within 2-3 weeks for consumption. This process favours growth of lactic acid bacteria (LAB) that convert fermentable carbohydrates to organic acids with concomitant reduction in pH. Reduced pH, salt and spices or sugar inhibit growth of spoilage microorganisms. Carefully fermented and hygienically preserved pickles may have shelf life of 2-3 years. LAB may have probiotic character (Gupta and Sharma, 2017; Gautam *et al*., 2004). In West Bengal, different varieties of pickles, locally called achar, is a delicacy along with staple foodstuff such as rice, chapathi, snacks etc. However, no work on the fermenting microorganisms present in pickles from West Bengal and their characters is available. This work aims at isolation, characterization and identification of predominat microorganisms present in pickles and to explore their probiotic potential.

MATERIALS AND METHODS

The culture media and sampling bags used were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. The genomic DNA of each bacterial strain was extracted and purified using Chromous Genomic DNA isolation kit.

Sampling

A total of 50 samples of vegetable pickles were procured randomly from retail shops, mobile street vendors as well as collected from households of Kolkata, India and adjoining areas in sterile sampling bags. Samples were immediately transferred to the laboratory in ice box for bacterial isolation.

Isolation of Lactic acid bacteria

1g of sample was added to 9ml of sterile physiological saline (0.85% NaCl solution) and mixed thoroughly with vigorous shaking at 100 rpm in rotary shaker for 2 min. Serial dilutions were prepared with the same diluents and one ml of appropriately diluted suspensions was then pour plated in MRS Lactobacillus agar medium supplemented with 0.5% (w/v) $CaCO_3$ and 0.05% (w/v) L-cysteine-hydrochloride (MRSC agar) followed by MRSC agar overlay and incubated under anaerobic condition using a candle extinction jar with a moistened filter paper to provide a CO_2-enriched, water-vapour saturated atmosphere at 30°C for 48-72h. Colonies appearing on the plates were enumerated, categorized into different colony morphotypes and purified by streaking at least twice on MRSC agar. The individual bacterial colonies were stored in sterile MRS broth at 4°C for preliminary screening.

Preliminary screening of lactic acid bacteria

From each colony morphotypes single isolate was randomly chosen for preliminary screening of lactic acid bacteria (LAB) based on Gram staining, cell morphology, sporogenicity and catalase test (Harrigan and MacCance, 1976). Gram- positive, non-spore forming and catalase-negative strains were considered as LAB. Isolates were stored in sterile MRS broth at 4°C for study of antimicrobial activity.

Phenotypic characterization

Growth at 10, 15 and 45°C in MRS both was checked for 5 days. Growth at different pHs of 3.9 and 9.6 for 3 days was tested in MRS broth. NaCl tolerance was studied by incubating the isolates in different concentration of NaCl (6.5, 10 and 18%) amended MRS broth at 30°C for 3 days and observed for turbidity (Mandal *et al*., 2011). The isolates were tested for hydrolysis of arginine, homo/hetero lactic fermentation, IMViC test and nitrate reduction (Harrigan and Mac Cance, 1976).

Study of sugar fermentation pattern

Sugar fermentation pattern was tested by inoculating tubes of 5ml sugar basal broth (Garvie, 1984) containing 2% sugar with inverted Durham's tube and incubating at 30°C for 5 days.

Yellowing of the suspension and accumulation of gas in the Durham's tube indicated acid and gas production.

Genotypic characterization of selected isolates

One each representative of rod (O11) and coccus (Ea3) shaped isolates were selected to identify and assign closest taxonomic position based on 16S rDNA PCR amplification and sequencing. The 16S rDNA gene amplification of O11 was done using eubacterial forward primer FC27 (5'-AGA GTT TGA TCC TGG CTC AG-3) and reverse primer 1492R (5'-TAC GGC TAC CTT GTT ACG ACT T-3'). Forward primer: 5'-AGHGTBTGHTCMTGNCTCAS -3' and reverse primer: 5'- TRCGGYTMCCTTGTWHCGACTH -3' were used for Ea3. DNA amplification was carried out in a 100 µl PCR mixture containing 1 µl DNA, 400 ng dNTPs (2.5mM each), 400 ng of each primer, 3 U of Taq DNA polymerase and 10 µl 10X Taq DNA polymerase assay buffer. Each PCR cycle consisted of an initial denaturation time of 5 min at 95°C followed by 35 cycles of amplification comprising a denaturation step for 30 sec at 94°C, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec. Reactions were completed with 7 min elongation at 72°C. PCR products were resolved by electrophoresis at a constant voltage (200 V) in 1 % (w/v) agarose. After purification, amplification products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3500 Genetic Analyzer. The nucleotide sequences of the 16S rRNA gene were analyzed and determined by the BLAST programme on the NCBI website to determine percent identity of the closest cultured strain.

Antibiotic sensitivity assay

Antibiotic susceptibility was tested using the disc agar diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar plate (4 mm thick). The result (average of 3 readings) was expressed as sensitive (S), intermediate (I), resistant (R).

Production of extracellular enzymes

Lactic acid bacterial cultures grown in MRS broth were tested for the production of enzymes viz.,
amylase, caseinase, lipase, gelatinase and DNase on starch agar, standard method caseinate agar, tributyrin agar base supplemented with 1.0% v/v tributyrin, MRS broth containing 1% gelatin and DNase agar with methyl green, respectively.

Antimicrobial activity of lactic acid bacteria

Antimicrobial activity of the isolates was initially checked by Agar-spot test (Schillinger and lücke, 1989). An aliquot of 2 µl of a 18 h old culture grown in MRS broth was spotted on MRS-0.2 agar (MRS broth containing only 0.2% glucose and 1.2% agar) and plates were incubated in a candle extinction jar at 30°C for 48 h to allow exhibition of antimicrobial compound. Cell suspensions of the indicator microorganisms prepared in Brain Heart Infusion (BHI) broth supplemented with 1% (w/v) agar, termed BHI soft agar was seeded with 18 h old 10^5 indicator organisms per ml. Seven ml of seeded BHI soft agar was overlaid on the plate on which the producer is grown. After incubation at 30°C for 18-24 h, plates were checked for zones of inhibition surrounding the producer colonies. Inhibition recorded as positive if the width of the clear zone around the colonies of the producer was 0.5 mm or larger.

RESULTS AND DISCUSSION

Altogether 50 samples belonging to 14 different types of pickles categorized on the basis of their principal ingredient (vegetables and fruits) were collected for analysis (Table 1). Of these, 8 pickles were home-made, 7 collected from mobile street vendors and rest 35 were purchased from retail shops. Some of the samples were oil based while others sugar based. All of the samples were acidic in nature excluding one mango pickle having neutral pH. pH of the samples ranged from 3.3 for plum and lemon pickles both collected from street vendors to 7.2 for a mango pickle of home-made type. Sixty percent of the samples had a pH ranging from 3.3-4.0 while 28% of the samples had pH 4.1-5.0. Both coccus and rod-shaped bacteria were observed in a single morphotype of colony. pH of the final product largely depends on types as well as concentration of the fermenting LAB. Viable count on Lactobacillus MRS agar ranged from $2.0x10^1$ for chilly pickle to $2.2x10^{11}$ for a plum pickle. Fifty-four percent of the samples contained LAB at a level upto 10^4cfu g^{-1} while 32% from 10^5-10^7cfu $g^{-1}$and 10% from 10^8-10^{10} cfu g^{-1}. Growth of fermenting LAB depends on environmental parameters such as nutrients, temperature of incubation, oxidation-reduction potential and pH of the fermenting media (Frazier and Westhoff, 2004). Salt concentration and incubation temperature have profound effects on acidity and flavor development in pickle fermentation (Fleming, 1982). Room temperature fermentation is found to be the best for organoleptically acceptable carrot pickle (Ramdus and Kulkarni, 1987).

One sample of elephant apple pickle having a pH of 3.7 contained LAB below detection limit (i.e., 10 cfu g^{-1}). This may be due to intentional chemical acidification without fermentation. Fermented vegetables are relished throughout the world like sauerkraut by German and Scandinavian, fermented olive by Mediterranean and pickled cucumber in USA. As the demand for pickle in the international market is very high indigenous pickling technology should be explored scientifically for controlled large scale production.

Table 1. Sampling of pickles from Kolkata and adjoining areas

Principal ingredient	No. of samples analyzed	Source*	pH	MRS count (cfu g^{-1})	Representative isolate
Chilly	5	HM(1), RS(4)	3.6-5.6	$2.0x10^1$-2.98 x 10^7	C1
Date	3	HM(1), RS(2)	3.9-4.1	$3.5x10^3$-$6.0x10^4$	D2
Elephant apple	3	RS(3)	3.7-4.0	$1.2x10^3$-$1.5x10^2$	Ea3
Garlic	2	RS(2)	5.2	$1.2x10^3$-$1.4x10^4$	G4
Indian gooseberry	5	HM(1), SV(1), RS(3)	3.8-5.6	$1.4x10^3$-$2.0x10^8$	Ig5
Jackfruit	4	HM(2), SV(2)	4.3-4.7	$2.8x10^2$-$4.4x10^6$	Ja6
June plum	2	RS(2)	3.8-4.0	$1.5x10^3$-$6.0x10^3$	Jp7
Lemon	5	SV(1), RS(4)	3.3-4.6	$2.5x10^2$-$1.0x10^9$	L8
Mango	8	HM(2), SV(1), RS(5)	3.7-7.2	$1.0x10^3$-$1.0x10^9$	Ma9
Mixed fruit	3	RS(3)	3.5-4.3	$3.0x10^1$-$2.5x10^6$	Mf10
Olive	3	HM(1), SV(1), RS(1)	3.7-3.9	$7.0x10^1$-$6.5x10^4$	O11
Plum	3	SV(1), RS(2)	3.3-4.2	$2.8x10^8$-$2.2x10^{11}$	P12
Tamarind	2	RS(2)	3.4-3.9	$5.0x10^5$-$7.7x10^6$	Ta13
Tomato	2	RS(2)	3.8-4.1	$5.0x10^5$x$5.1x10^6$	To14

*HM (Home-made)/ SV (Street vendor)/ RS (Retail shop)

Table 2. Colony morphology and representative LAB isolates from pickles

Colony morphotype	LAB isolate	
	rod singly or in pair	Coccus singly or in clump
2-3 mm dia., white, circular, translucent, surface convex, margin entire, colony surrounded by clear zone	C1, D2, Ja6, Jp7, L8, Ma9, Mf10, O11, P12, Ta13, To14	Ea3, G4, Ig5

Of all the isolates considered to be LAB, 14 isolates, one from each type of pickles, including 11 rods and 3 cocci were selected for biochemical characterization (Table 2 and 3). All the isolates were able to grow at both 10°C and 45°C. Both pH of 3.9 and 9.6 were favourable for their growth. Growth of all the isolates was observed when challenged against NaCl concentration of 4-18%. All the isolates except two cocci from elephant apple and garlic hydrolyzed arginine to produce ammonia. All the isolates were indole negative, methyl red positive and citrate negative. None the isolates produced extracellular enzymes viz., amylase, caseinase, lipase, gelatinase and DNase.

Table 3. Physiological and biochemical characterization of the representative LAB isolates from pickles

Character	Isolate													
	C1	**D2**	**Ea 3**	**G4**	**Ig5**	**Ja 6**	**Jp 7**	**L 8**	**Ma 9**	**Mf1 0**	**O11**	**P12**	**Ta13**	**To14**
Growth at 10 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 3.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 9.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 6.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 10% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 18% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Homo/Hetero	He	Ho	Ho	Ho	Ho	Ho	Ho	H o	Ho	Ho	Ho	Ho	Ho	Ho
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	+	-	+	-	+	-	-	+	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of extra cellular enzymes														
Amylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Caseinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sugar fermentation pattern (Table 4) of the isolates was tested using thirteen different sugars. Of the eleven rod shaped isolates, ten showed similar sugar fermentation pattern and produced acid without gas from all the sugar. Sugar fermentation patterns of the three cocci were different from rods as well as among themselves. Phylogenetic tree based on Neighbor Joining method generated after blasting the sequences of the isolates is presented in figure 1. The generated sequence (1439 bp) from O11 isolates showed 100% identity with published 16S rDNA sequences of cultured *Lactobacillus* strains viz., *L. plantarum* sub sp. *plantarum.*

Table 4. Study of sugar fermentation pattern of the representative LAB isolates from pickles

Sugar	Production of acid/gas*													
	C1	D2	Ea3	G4	Ig5	Ja6	Jp7	L8	Ma9	Mf10	O11	P12	Ta13	To14
L(+)Arabinose	w/-	+/-	+/-	+/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Cellobiose	+/-	+/-	w/-	w/-	w/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
Dextrose	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Fructose	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
Lactose	-/-	+/-	w/-	w/-	w/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
Maltose	+/-	+/-	w/-	w/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Mannitol	-/-	+/-	-/-	w/-	w/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Mannose	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D(-)Salicin	+/-	+/-	-/-	-/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Sorbitol	+/-	+/-	-/-	-/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
Sucrose	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D-Trehalose dihydrate	-/-	+/-	w/-	w/-	w/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-
D(+)Xylose	w/-	+/-	w/-	w/-	w/-	+/-	+/-	+/	+/-	+/-	+/-	+/-	+/-	+/-

*W, weakly positive; +, positive; -, negative

On the other hand, 1446 bp sequenced amplicon from Ea3 was found to be most similar to *Pediococcus acidilactici* and *P. lolii* 16S ribosomal RNA genes. Predominant LAB associated with vegetable fermentation of cabbage include *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Pediococcus pentosaceous* and *Lactobacillus plantarum* (Pederson, 1979). Traditionally fermented vegetables in India, Nepal and Bhutan are predominated by *Lactobacillus brevis*, *L. plantarum, L. curvatus*, *Pediococcus pentosaceus*, P. *acidilactici*, and *Leuconostoc fallax* (Tamang *et al*., 2005). Lactobacilli are most extensively used food grade bacteria with probiotic potential. Fermented fruits and vegetables contain different types of prebiotic compounds that can facilitate growth of probiotic strains of LAB (Dwivedi *et al*., 2014).

Antibiotic resistance pattern (Table 5) of the fourteen isolates against thirteen different antibiotics shows that most of the isolates are sensitive or intermediate against the antibiotics tested. 100% sensitivity was found against three protein synthesis inhibitors viz., azithromycin, chloramphenicol and erythromycin and one nucleic acid synthesis inhibitor viz., levofloxacin. None of the isolates was resistant to bacitracin, kanamycin and streptomycin. Only three antibiotics viz., penicillin-G, amikacin and tetracyclin showed resistance to more than fifty percent isolates.

All the lactic acid bacteria showed antimicrobial activity by agar spot method against the three indicator organisms of both gram positive and gram-negative type indicated by inhibition zones ranging from 12 mm to 45 mm (Figure 2a, 2b, 2c). Diameter of inhibition zone against *B. cereus, E. coli and S.* Typhimurium ranged from 12 to 34 mm, 16-40 mm and 30-45 mm, respectively. Inhibitory activity was higher against gram negative organisms. Antimicrobial activity observed in this method may be due to combination of organic acids viz., acetic acid, lactic acid, succinic acid or hydrogenperoxide, antimicrobial proteins etc (Gautam *et al*., 2014).

Figure 1. Phylogenetic tree of O11 and Ea3 isolate

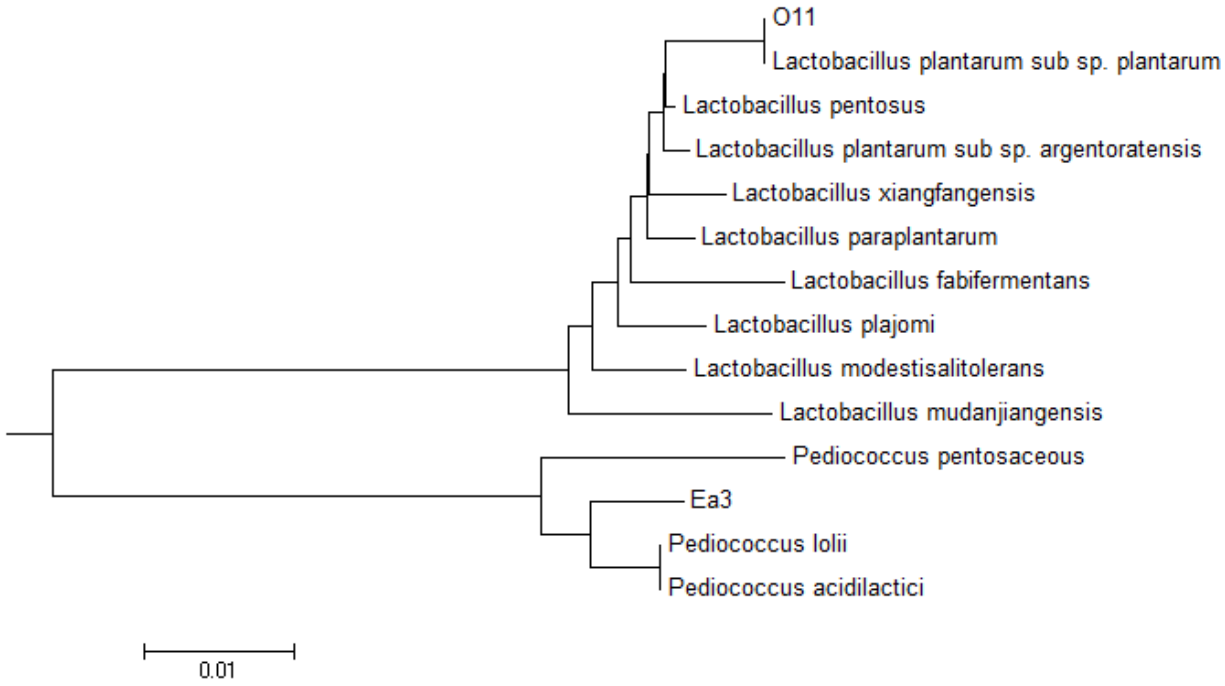
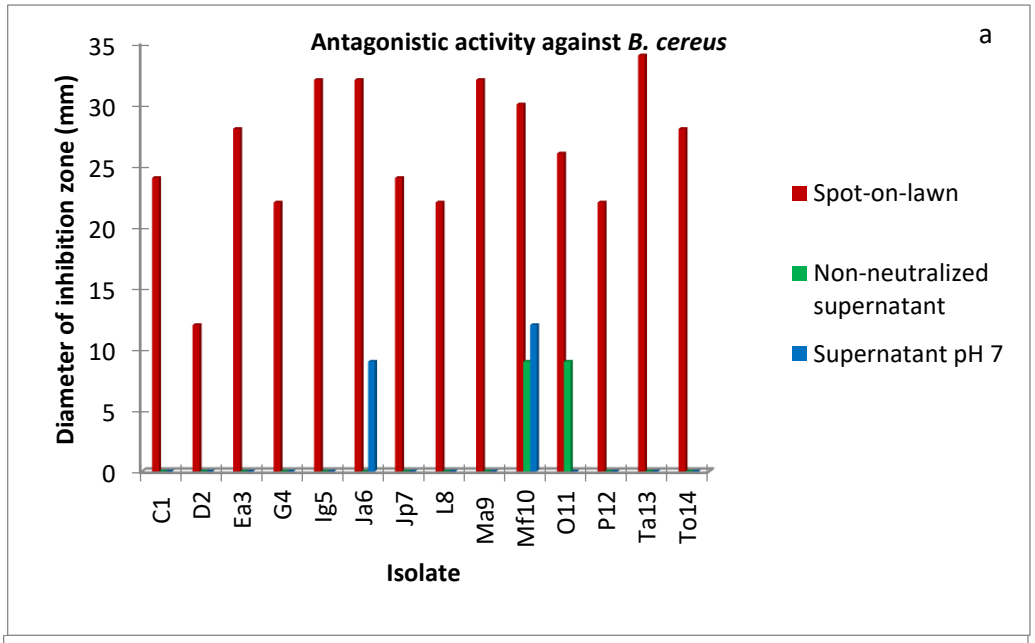


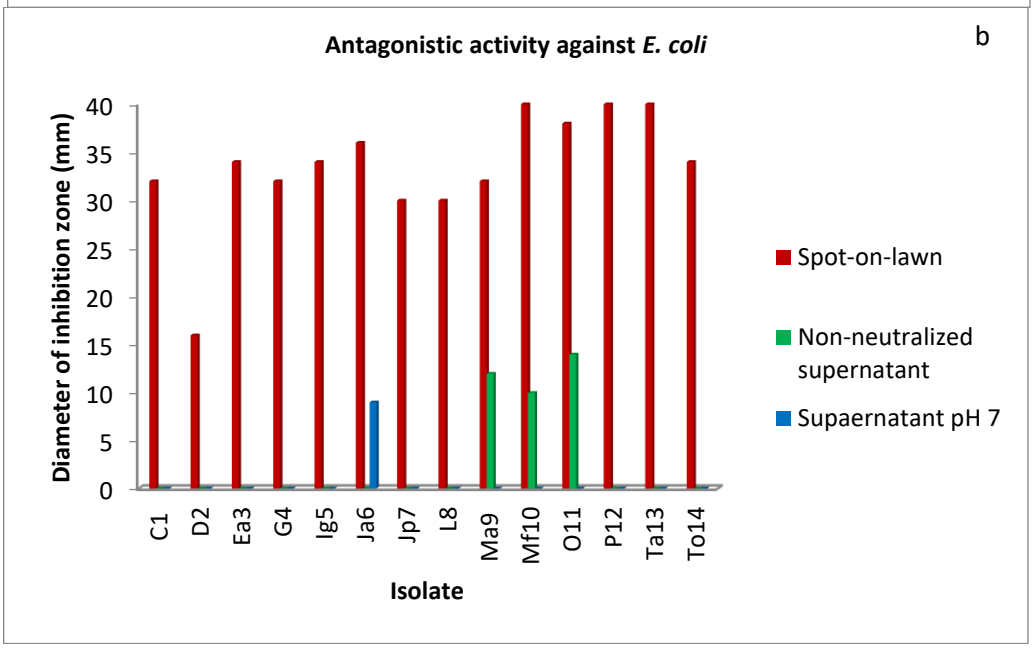
Table 5. Antibiotic resistance pattern of the representative LAB isolates

Antibiotic	**Percentage of isolates***		
	S	I	R
Cell wall synthesis inhibitor			
Bacitracin (10U)	29	71	0
Penicillin-G (10U)	0	0	100
Cell membrane synthesis inhibitor			
Polymyxin-B (300U)	86	0	14
Protein synthesis inhibitor			
Amikacin (30µg)	0	21	79
Azithromycin (15µg)	100	0	0
Chloramphenicol (30µg)	100	0	0
Erythromycin (15µg)	100	0	0
Kanamycin (30µg)	0	100	0
Streptomycin (10µg)	50	50	0
Tetracyclin (30µg)	0	7	93
Nucleic acid synthesis inhibitor			
Ciprofloxacin (10µg)	0	57	43
Levofloxacin (5µg)	100	0	0
Ofloxacin (5µg)	7	64	29

*S, Sensitive; I, Intermediate; R, Resistant

On further study using non-neutralized (pH ca. 5.0) cell free supernatant of lactic acid bacteria by agar well diffusion method, only 2, 3 and 4 isolates showed inhibitory activity against *B. cereus*, *E. coli* and *S.* Typhimurium, respectively. Variation in inhibitory activity using supernatant indicates that reduction in pH is not the sole reason of inhibition. On further study using cell free supernatant of pH 7, inhibitory activity against *B. cereus*, *E. coli* and *S.* Typhimurium was observed by only 2 (Ja6, Mf10), 1(Ja6) and 1 (Ja6) isolates, respectively. Ja6 isolate showed inhibitory activity at pH 7 but not at pH 5. This confirms that some non-acidic metabolites may be active at neutral pH to inhibit the growth of the indicator organisms. Antimicrobial activity of LAB isolated from vegetable pickles has also been reported by Hartayanie *et al*., (2016) and Wakil *et al*., (2014).





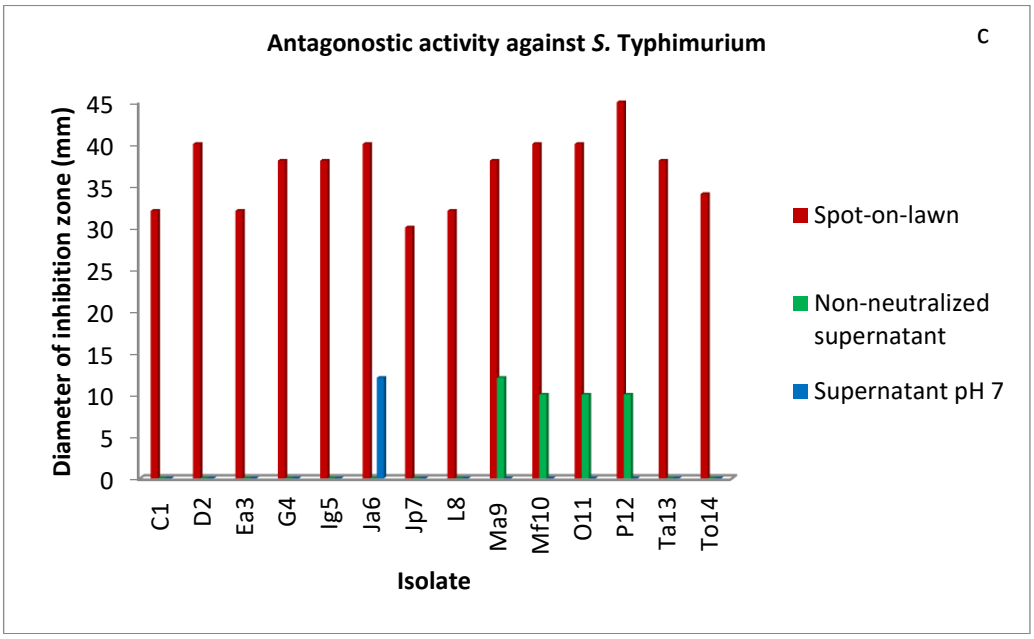


Figure 2. Antagonistic activity of the lactic acid bacterial isolates against *B. cereus* (2a), *E. coli* (2b) and *S.* Typhimurium (2c)

Conclusion

Production of organic acids during fermentation of vegetables and fruits reduce pH of the pickle inhibiting the growth of undesirable microorganisms. Indian pickles are predominantly fermented by *Lactobacillus plantarum* and *Pediococcus acidilactici*. The LAB have antimicrobial activity against both gram positive and gram negative bacteria conferring upon them probiotic potential. Further research to explore their bacteriocin production is important.

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