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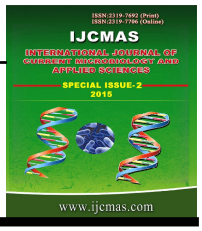

Original Research Article

Inhibitory activity of Lactic acid bacteria against isolated pathogens and spoilage organisms associated with fresh meat

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Keywords

Lactic acid bacteria as *Pediococcus acidilactici* strain CSI29MX, LAB-B as *Pediococcus parvulus* strain, Food borne pathogens, Inhibitory activity, Meat.

A B S T R A C T

The use of lactic acid bacteria (LAB) as protective cultures isolated from idli batter has potential application for biopreservation of perishable food meat. In a study a total of 100 presumptive LAB isolates were collected from idli batter from different sources. Out of 100 isolates 20 % showed antimicrobial activity against pathogens and spoilage organisms associated with meat and genetically closely related organism *L. acidophilus* MTCC 10307. The highest potential LAB-A, LAB-B and LAB-C were selected and characterized morphologically and biochemically. 16S ribosomal RNA sequencing of isolates were done to identify the isolates. LAB-A was identified as *Pediococcus acidilactici* strain CSI29MX, LAB-B as *Pediococcus parvulus* strain MF233 while LAB-C as *Pediococcus pentosaceus* strain QN1D. Pathogenic and spoilage organisms *S. aureus*, *E. coli*, *S. typhi*, *B. cereus*, *P. vulgaris* and *P. aeruginosa* were isolated from ten meat samples and identified. The inhibitory activity of LAB isolates was tested against the food borne pathogens. The present results demonstrated that all the three isolates were equally antagonistic against both the Gram positive organisms, *Staphylococcus aureus* and *Bacillus cereus* as well as Gram negative organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Proteus vulgaris.*

Introduction

The antibacterial spectrum of Lactic acid bacteria frequently includes spoilage and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* as well as Gram negative bacteria such as *E. coli* and *Salmonella* (Stevens *et al.*, 1991). The anti-microbial properties of LAB have enabled the extension of the shelf life of many foods through fermentation processes. The inhibition of food spoilage microbes could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins (Atrih *et al.*, 1993). Many species of *Lactobacillus*, used in the manufacture of fermented dairy products,

inhibit the growth of other bacteria including the intestinal pathogens and spoilage organisms by producing anti-bacterial compounds or bacteriocins. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity against those bacteria which are closely related to the producer strain (Klaenhammer, 1988).

The bacteriocins produced by Gram positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative and therapeutic potentials (Galvez *et al.,* 2008; Jack *et al.,* 1995). Considering this quality, there has been an increased concern in recent years on usage of bacteriocins due to the wide spread over-prescribing of antibiotics and consequent increased development of antibiotic resistance.

Material and Methods

The indicator organisms *L. acidophilus* MTCC 10307 was procured from Microbial Type Culture Collection (MTCC) Chandigarh, India. Bacteriocinogenic LAB were isolated from idli batter. Food borne pathogens isolated from meat obtained from local market. Culture media and chemicals were obtained from HI media Mumbai.

Isolation of lactic acid bacteria

The highest potential Bacteriocinogenic Lactic acid bacteria LAB-A, LAB-B and LAB-C were isolated from idli batter (Khandare and Patil, 2014). 16S ribosomal RNA sequencing of isolates were done to identify the isolates. LAB-A was identified as *Pediococcus acidilactici* strain CSI29MX, LAB-B as *Pediococcus parvulus* strain MF233 while LAB-C as *Pediococcus pentosaceus* strain QN1D.

Isolation of pathogens and spoilage organisms from meat

Pathogens and spoilage organisms were isolated from perishable food meat. Samples were examined for initial pH, Total viable count, proteolytic organisms, pathogens and spoilage organisms.

10 gms of meat samples were homogenized with blender. 1:10 dilutions were prepared in 90 ml of 0.85% sterile physiological saline. Serial dilutions were made upto 10^{-7} to obtain different number of bacteria. The diluted samples from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were inoculated in differential and selective media viz. Eosine methylene blue agar, Cystine Lactose Electrolite Deficient medium, Mannitol salt agar, Pseudomonas isolation agar, Salmonella Shigella agar and Mannitol Egg Yolk Polymixin agar, and incubated at 37^0C for 24 h.

Identification of pathogens and spoilage organisms on the basis of biochemical tests

The colonies of pathogens and spoilage organisms (PSO) obtained on selective and differential media were identified on the basis of Gram staining, Sugar fermentation, IMViC test, H_2S production, production of enzyme coagulase, urease and gelatinase. Christen Gram procedure was used for staining. The PSO were tested for their carbohydrate fermentation ability by inoculating the LAB in glucose, lactose and mannitol broth. PSO were tested for Indole, methyl red, Voges Prousker, citrate utilization and H_2S production. The PSO were studied for production of enzyme, urease and gelatinase (Aneja, 2003) and coagulase (Ananthanarayan and Paniker 2013).

Antimicrobial activity of bacteriocinogenic LAB against food borne pathogens and spoilage organisms

The antimicrobial activity of bacteriocinogenic LAB isolates and standard strain of *L. acidophilus* MTCC 10307 was tested against PSO obtained from perishable food meat. Cell free supernatant (CFS) was obtained by centrifugation at 12000 rpm, at 4^0C for 13 min. To eliminate the possible inhibitory effect of either hydrogen peroxide or lactic acid on the indicator strain, pH was neutralized and catalase treated supernatant of overnight culture was used (Ogunbanwo *et al.*, 2003). Supernatant was sterilized by filtration through 0.22 mm millipore membrane filter. 500 µl of 24 h broth culture of *S. aureus*, *E. coli. S. typhi. B. cereus P. vulgaris* and food spoilage organism *P. aeruginosa* was seeded on Muller- Hinton agar plate and *L. acidophilus* MTCC 10307 on MRS agar. The wells of 2 mm were bored and to each well 20 µl of CFS was added and incubated at 37 ^{0}C for 24 h. Antimicrobial activity was evaluated by observing zone of inhibition (Narayanapillai *et al.*, 2012). The antimicrobial activity of LAB isolates was compared with standard strain of *L. acidophilus* MTCC 10307 obtained from MTCC Chandigarh.

Results and Discussion

Ten meat samples were examined for initial pH, total viable count, proteolytic organisms and PSO. The pH of the sample ranges from 6.8 -7.2. Total viable count was 7.0 to 10.2 $x10^4$ cfu / ml, proteolytic organisms were 2.5 to 4.5$x10^4$ cfu /ml whereas PSO were 0.2 to 3.5 $x10^3$ cfu/ml. (Table 1).

The morphological and biochemical characteristics of isolates of meat were studied. On Eosine Methylene Blue agar the colony with green metallic sheen was observed which showed Gram negative, sluggishly motile rods fermenting glucose, lactose and mannitol with production of acid and gas. Indole, methyl red tests were positive and Voges Proskauer, citrate and H_2S tests were negative.

The isolated green coloured colonies on Cystine Lactose Electrolyte Deficient agar showed Gram negative, motile rods fermenting glucose and mannitol with production of acid and gas, lactose was not fermented. Indole, methyl red, citrate, H_2S and protease tests were positive while Voges Proskauer test was negative. On Mannitol salt agar pink coloured colonies were observed which showed Gram positive cocci, non motile, fermenting glucose, lactose and mannitol with production of only acid. Indole and citrate tests were negative and methyl red, Voges Proskauer, coagulase tests were positive.

Growth on Pseudomonas Isolation agar, showed white greenish colony found to be Gram negative, actively motile rods, fermenting glucose with production of acid while lactose and mannitol were not fermented. Indole, methyl red, Voges Proskauer, H_2S test were negative, while citrate test was possitive. No enzyme production was observed. On Salmonella Shigella agar, black colony was observed which showed, Gram negative, actively motile, rod shaped organisms fermenting glucose and mannitol with production of acid and gas but no lactose fermentation. Methyl red, citrate, H_2S test were positive, while indole,Voges Proskauer test were negative. On Mannitol Egg Yolk Polymyxin agar the pink orange colonies was formed that showed Gram positive, motile rods which ferments glucose with production of only acid. Mannitol was not fermented. While gelatinase and Voges Proskauer test were positive (Table 2, Table 3).

The antimicrobial properties of bacteriocinogenic *Pediococcus acidilactici* CSI29MX, *Pediococcus parvulus* MF233 and *Pediococcus pentosaceus* strain QN1D were tested against six food-borne pathogenic and spoilage bacteria namely *E. coli, S. aureus, B. cereus, P. vulgaris, S. typhi, P. aeruginosa* and were compared with *L. acidophilus* MTCC 10307. The results of antimicrobial activity demonstrated by the isolates in terms of diameter of the zone of inhibition (ZOI) were shown in Fig 1. A diameter >1mm around the well was considered as a positive result. In the present study highest ZOI has been shown by *Pediococcus acidilactici* CSI29MX viz 15 $\pm$ 1.0 mm while *Pediococcus parvulus* MF233 and *Pediococcus pentosaceus* QN1D both demonstrated 14 $\pm$ 0.8 mm ZOI against Gram negative pathogen *E. coli*. *Pediococcus acidilactici* CSI29MX demonstrated 14 $\pm$0.6 mm ZOI against both *S. aureus* and *B. cereus. Pediococcus pentosaceus* QN1D showed highest activity against Gram positive organisms *S. aureus* and *B. cereus* with a ZOI of 16 $\pm$ 0.1 mm and 16 $\pm$ 0.4 mm respectively. *Pediococcus pentosaceus* QN1D showed ZOI 15 $\pm$ 0.1 mm against *P.aeruginosa* while lowest against *P. vulgaris* with ZOI 13 $\pm$ 0.6 mm. *Pediococcus parvulus* MF233 demonstrated the activity with 14 $\pm$ 0.6 mm and 14 $\pm$ 0.5 mm ZOI against *S. aureus* and *B. cereus* respectively. *L. acidophilus* MTCC 10307 demonstrated lowest antimicrobial activity with 12 $\pm$ 0.6 mm and 12 $\pm$ 0.7 mm ZOI against *S. aureus* and *B. cereus* respectively.

In industrialized countries, the percentage of the population suffering from food borne disease each year has been reported to be up to 30%. The epidemiology of foodborne diseases has changed in the last two decades partly because newly recognized pathogens emerge and previously recognized pathogens increase in occurrence or become associated with food or new food vehicles (Meng *et al.*, 1997).

Bodhidatta *et al.* (2013) isolated food borne pathogens from raw chicken, pork and fish. In this study, only 97% of raw food samples were found to contain bacterial enteric pathogens. For Bacterial identification the bacteria was cultured on enrichment medium followed by conventional phenotypic tests. In our study 100 % food samples were found to contain pathogens and spoilage organisms, They were identified by morphology, cultural characteristics and phenotypic tests The method of identification of present study agreed with the method of Bodhidatta used for identification of isolates.

Kazemipoor *et al.* (2012) had earlier reported greater activity of isolate MF15 against *E. coli* showed activity with a ZOI of 12 ± 0.8 mm while MF6 showed activity with a ZOI of 20 ± 1.3 mm. In the present study highest ZOI has been shown by *Pediococcus acidilactici* CSI29MX viz 15 $\pm$ 1.0 mm while *Pediococcus parvulus* MF233 and *Pediococcus pentosaceus* QN1D demonstrated 14 $\pm$ 0.8 mm ZOI against Gram negative pathogen *E. coli*. So the inhibitory spectra of *Pediococcus acidilactici* CSI29MX of present study was found to be more than isolate M6 while less than MF15. Kivinac (2011), isolated the LAB *Leuconostoc citreum* KB2, from boza, a drink from Turkey, which demonstrate antimicrobial activity against *B. cereus* 16.1 to 22 mm as ZOI, while another strain *Lactobacillus brevis* KB12 (1) showed 11.1 to 16 mm and 16.1 to 22 mm ZOI against *S aureus* and *B. subtilis* respectively. The *Leuconostoc citreum* KB2 and *Lactobacillus brevis* KB12 (1) strain was found to have more potential of bacteriocin production than our strains, since we got lower ZOI against *B. cereus* and *S. aureus*.

Table.1 Physicochemical properties, pathogens and spoilage organisms of meat

Sample	Consistency Colour odour	Initial pH	TVC $x10^4$ cfu/ml	*E. coli* $x10^3$ cfu /ml	*S. aureus* $x10^3$ cfu /ml	*S. typhi* $x10^3$ cfu/ml	*P. vulgaris* $x10^3$ cfu /ml	*P. aeruginosa* $x10^3$ cfu /ml	*B. cereus* $x10^3$ cfu /ml	Proteolytic organisms $x10^4$cfu /ml
M1	Fresh, reddish odourless	7.2	9.5	3	-	1.8	-	-	-	4
M2	Fresh, reddish pink, odourless	6.8	7	-	-	1.2	-	2.4	-	3.9
M3	Fresh, Red, Bloody, odourless	7	9	2.6	2.1	-	-	-	-	4.2
M4	Fresh, Red, odourless	6.9	10.2	2.9	-	-	-	3.2	1.8	4.5
M5	Fresh, Red bloody, odourless	7	10	-	2.8	1.3	2.2	-	0.6	4.5
M6	Fresh, Red, odourless	7.2	9.5	-	2.8	1.4	2.0	3.5	0.2	4.5
M7	Fresh, reddish odourless	6.8	8	1.6	2.5	-	-	-	-	3.0
M8	Fresh, Red Bloody, odourless	7	6.8	-	-	-	1.5	3.0	-	3.6
M9	Fresh, Red,odourless	7	8.9	-	-	0.4	1.1	2.4	-	3.4
M10	Fresh, reddish odourless	6.8	9	0.9	-	-	-	2.0	-	2.5

Table.2 Colony characteristics of pathogens and spoilage organisms obtained from meat

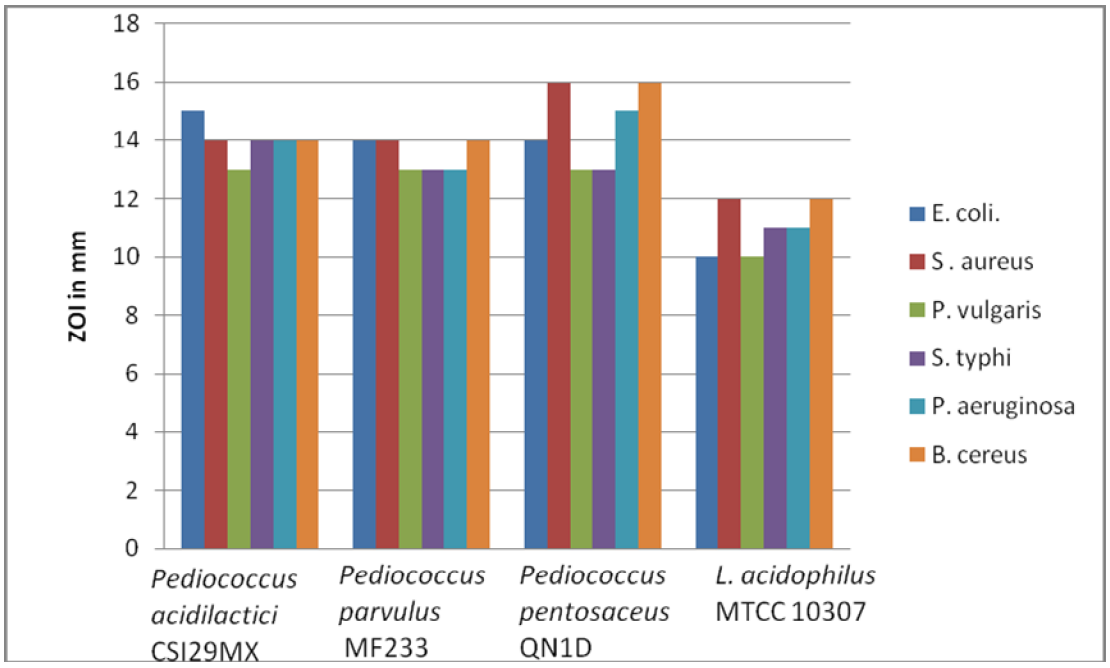
	Meat samples									
Media	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
EMB Agar	Green metallic sheen colony	-	Green metallic sheen colony	Green metallic sheen colony	-	-	Green metallic sheen colony	-	-	Green metallic sheen colony
CLED agar	-	-	Green colony	-	Green colony	Green colony	-	Green colony	Green colony	-
MS agar	-	-	Small pink colony	-	Small pink colony	Small pink colony	Small pink colony	-	-	-
PI agar	-	Small greenish colony	-	Small greenish colony	-	Small greenish colony	-	Small greenish colony	Small greenish colony	Small greenish colony
SS agar	Black centered colony	Black centered colony	-		Black centered colony	Black centered colony	-	-	Black centered colony	-
MYP Agar	-	-	-	Pink orange	Pink orange	Pink orange	-	-	-	-

Table.3 Biochemical characteristics of pathogens and spoilage organisms obtained from meat

Colony on	Gram staining	Motility	Sugar fermentation			Enzyme production	H_2S	Indole	M.R. Test	VP Test	Citrate Test
			Glucose	Lactose	Mannitol						
EMB Agar	Gram negative, rods	Sluggishly motile	AG	AG	AG	_	_	+	+	_	_
CLED Agar	Gram negative, rods	Highly motile	AG	_	AG	Protease	+	+	+	_	+
MS agar	Gram possitive cocci	Non motile	A	A	A	Coagulase	ND	_	+	+	_
PI agar	Gram negative, rods	Actively motile	A	_	_	_	_	_	_	_	+
SS agar	Gram negative, rods	Highly motile	AG	_	AG	_	+	_	+	_	+
MYP agar	Gram positive, rods	Motile	A	ND	_	Gelatinase		ND	ND	+	ND

ND –Not determined, AG-Acid and gas production, A-Acid production, + Positive, - Negative

Fig.1 Zone of inhibition shown by LAB isolates against pathogens and spoilage organisms



The present results demonstrated that all the three isolates were equally antagonistic against both the Gram positive as well as Gram negative organisms. Also all the LAB isolates demonstrated higher antimicrobial activity against pathogens and spoilage organisms compared with the activity of indicator strain *L. acidophilus* MTCC 10307.

Narayanapillai *et al.* (2012) isolated LAB from chick intestine showed production of antimicrobial compounds against *E. coli, P. aeruginosa, S. typhi, S. aureus, B. cereus, P. mirabilis* and *K. pneumoniae*. This result showed similarity with the present study. The isolates of present study demonstrated the antimicrobial activity against *E. coli, P. aeruginosa, S. typhi, S. aureus,* and *B. cereus*.

According to Zottola *et al.* (1994) the effect of nisin produced by LAB *in situ* against Gram positive microorganisms like *Listeria, Staphylococcus* and *Clostridium* was demonstrated and there are only few reports on Gram negatives. The effect of bacteriocin produced *in situ* against Gram negative is of greater importance since most of the food borne pathogens are Gram negatives. In the present study the LAB isolated showed antimicrobial activity against Gram negative organism *E. coli., P. aeruginosa, P. vulgaris* and *S. typhi*. It is however difficult to comment on the reason for this variability in the antimicrobial property amongst the isolates since each one was different from the other.

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