Letter to the Editor

Antimicrobial Profile of Lactic Acid Bacteria Isolated from Vegetables and Indigenous Fermented Foods of India against Clinical Pathogens Using Microdilution Method

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In dairy and food industries lactic acid bacteria (LAB) have been used in form of starter culture that plays vital role in fermentation; as flavouring and texturizing or as preservative agents. There is increasing evidence that *lactobacilli* which inhabit the gastrointestinal tract develop antimicrobial activities and participate in the host's defence system[1]. During fermentation, most of the LAB produces a number of different compounds like organic acids, hydrogen peroxide, diacetyl, acetaldehyde, carbon dioxide, polysaccharides, and proteinaceous compounds called bacteriocins or bacteriocinogenic peptides[2-3]. Such metabolites exert antimicrobial activity and are found to inhibit spoilage causing and/or disease causing bacteria and thus help to maintain and preserve the nutritive qualities of foods for an extended shelf life. Because of 'Generally regarded as safe' (GRAS) status, the use of LAB or their metabolites, as a natal preservative in food has gained much importance in the recent years.

Enormous studies dealed with the production of antimicrobial substances from LAB because it is one of important criteria to wield probiotic properties. Bacteriocins or antimicrobial peptides produced by LAB such as *Lactobacillus*, *Leuconostocs*, *Pediococcus*, and *Lactococcus* species have been frequently reported in many studies, although very rare information is available for the genus *Weissella*[4]. LAB commonly produce antimicrobial substance(s) which are active against the homologous strain, but some of the LAB strains often produce microbicidal substances which are effective against intestinal pathogens and other microbes[1]. Very few bacteriocins have been reported to inhibit Gram-negative bacteria due to their narrow spectrum of action. Such characteristic often limits the potential of bacteriocins in food preservation and safety, and hence, the search for bacteriocin active against both Gram-positive and Gram-negative bacteria is worthwhile.

Earlier 17 LAB strains were isolated from vegetables and indigenous fermented foods of India based on their ability to produce exopolysaccharide (EPS)[4]. They were phenotypically and biochemically studied and characterized genotypically upto genus and species level by using 16S rRNA gene sequencing. The GenBank accession numbers for reference 16S rRNA gene sequences are shown in Table 1. As indicated, the isolates mainly belonged to the genera *Lactobacillus*, *Weissella,* and *Pediococcus.* Some of the isolates were shown to possess possible probiotic characteristics including resistance to low pH and to bile salts, bile salt hydrolase activity and antibiotic susceptibility showing a putative potential as probiotic strains. Antagonistic activity of LAB is an essential trait to prove their probiotic candidature and hence, the present research was aimed to screen and explore their antibacterial prospective against some common bacterial pathogens.

The pathogens were obtained from the Department of Medical Microbiology, Lund University, Lund (Sweden). Among the pathogenic bacteria, clinical isolates of *E. coli*-ESBL and Methicillin-resistant *Staphylococcus aureus* (MRSA); *Yersinia enterocolitica* CCUG 31004, *Salmonella typhimurium* CCUG 11732, *Enterococcus faecalis* CCUG 9997 (Culture collection of University of Gothenberg) and *Campylobacter jejuni* ATCC 33560 were used, considering their significant role as pathogens in various food products.

All strains of LAB were maintained and stored at -20 °C as stock cultures in de Man- Rogosa-Sharpe (MRS) broth (Merck) containing 10% (v/v) glycerol whereas all pathogens were propagated in brain heart infusion (BHI) broth (Oxoid) and maintained as frozen stocks at -20 °C in BHI broth containing 10% (v/v) glycerol. Before use, frozen cultures were plated onto MRS agar or BHI agar followed by

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two successive transfers into the respective liquid media.

During screening, the LAB isolates were tested against non pathogenic strain of E. coli K12 by using well-diffusion. About 8 mm diameter wells were punched in solidified plates of Luria bertani (LB) agar containing 50 µL overnight culture of indicator strain and filled with 100 µL of cell-free supernatant (CFS), obtained by centrifugation of the cultures at 4000 g, 15 min and filter-sterilization through 0.22 µmol/L filter (Acrodisc, USA). The plates were incubated at 37 °C for 24 h and subsequently observed for the zone of inhibition. Total eight strains showing higher antagonistic activities i.e. two strains of each *L. plantarum* (86 & AD29), *L. fermentum* (AI2 & AI3), *W. cibaria* (92 & 142) and one strain of each *W. confusa* AI10 and *P. parvulus* AI1 in well diffusion method were further selected to analyse their antagonistic behaviour against six different Gram-positive and Gram-negative clinical pathogens in microtiter plates.

For further confirmation, the microdilution method of MCVay & Rolfe[5] was employed to determine the level of antimicrobial spectrum of LAB isolates with few modifications by using 96 well microtiter plates. Initially, the CFS was obtained by harvesting 24 h old cultures from MRS broth by centrifugation at 4000 g, 15 min at 4 °C and then filter sterilized through 0.22 µmol/L filter. The CFS was serially diluted using BHI broth to prepare 1:1, 1:10, and 1:100 dilutions. From each dilution, 100 µL vol. was added in to the 96 well microtiter plate in triplicate and allowed to equilibrate at room temperature for 15 min. On the other side, pathogens were grown on blood agar plates for 24 h at 37 °C in aerobic or microaerophilic conditions, harvested and washed twice and finally suspended in sterile phosphate buffer saline (pH 7.2). From that, 10 µL of each pathogen adjusted to an A_{620}=0.4 (in BHI broth) was added to the wells of microtiter plate previously equilibrated with CFS of the LAB strains and was incubated overnight at 37 °C in plastic box. OD_{595} nm was measured by using iMark™ Microplate Reader (BIO-RAD) and results were recorded to find out percentage of growth inhibition of each pathogen with reference to growth in respective pathogen control.

Results indicated that the inhibitory activities of each LAB strains were very distinct towards all six pathogens (Figure 1a and Figure 1b). It was observed that in the fifty percentages diluted CFS (1:1 dilution) all LAB strains exhibited on an average 80%-90% growth reduction towards all pathogens except the strain *P. parvulus* AI1. The pediococci isolate exhibited less than 40% growth inhibition against *S. aureus* and about 60%-70% towards the other pathogenic bacteria. Other reported studies indicates the inefficiency of Pediococci to inhibit most of the clinical pathogens[3]. With respect to higher dilutions

Table 1. Screening of LAB Isolates for Antimicrobial Activity by Well Diffusion Assay against *E. coli* K12

Isolate Code	Source of Isolation	Similarity Based on 16S rRNA	Gene Accession Number	Zone of Inhibition (mm)
AD1	Dahi	*L. fermentum*	JN792470	17.2±0.76
AI2	dhokla batter	*L. fermentum*	JN792468	20.3±0.58
AI3	dhokla batter	*L. fermentum*	JN792457	16.2±1.04
AV2	fermented cabbage	*L. fermentum*	JN792461	23.2±0.76
AV3	Carrot	*L. fermentum*	JN792462	22.8±0.76
AV4	Cabbage	*L. fermentum*	JN792463	20.3±0.76
138	fresh turmeric	*L. fermentum*	JN792459	20.2±0.76
AD29	Dahi	*L. plantarum*	JN792465	23.2±0.76
86	Dahi	*L. plantarum*	JN792454	22.2±0.76
AI10	idli batter	*W. confusa*	JN792460	21.8±1.26
AV1	fermented cabbage	*W. cibaria*	JN792467	14.5±0.50
85	Dahi	*W. cibaria*	JN792458	18.8±1.04
92	idli batter	*W. cibaria*	JN792466	23.2±0.76
142	Cucumber	*W. cibaria*	JN792456	23.5±1.32
145	Cabbage	*W. cibaria*	JN792455	20.5±0.50
AI1	idli batter	*P. parvulus*	JN792469	19.7±1.04
AV5	Tomato	*P. parvulus*	JN792464	20.2±0.76

Note. ±: Standard deviation, *n*=3.

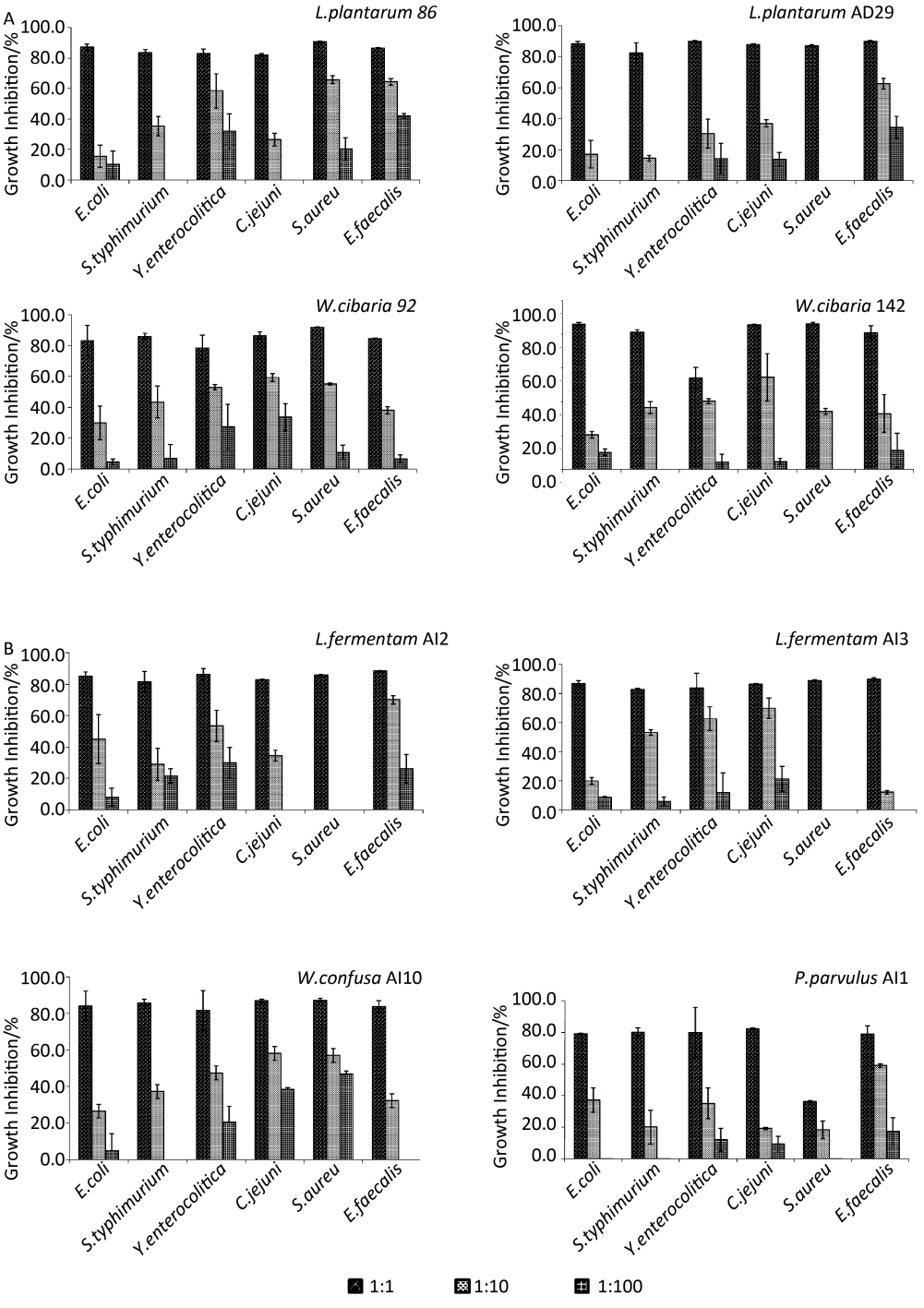

Figure 1. A) Antibacterial activity from cell free supernatant of isolated LAB strains against six clinical pathogens through microdilution method; B) Antibacterial activity from cell free supernatant of isolated LAB strains against six clinical pathogens through microdilution method.

(1:10 and 1:100), the growth of each pathogenic strain was successively increasing due to the decrease in the amount of CFS added in each well. In the tenfold (1:10) diluted CFS sample, *L. plantarum* 86, both the strains of *W. cibaria* 92 & 142; and *W. confusa* AI10 showed effective reduction in the growth of all pathogens, but it was not much impressive for the rest LAB strains. In higher (either

1:10 or 1:100 folds) dilutions, the microbicidal effect of pH or organic acids get eliminated and thus the reduction in the growth of pathogens indicates the presence of some other antimicrobial compounds. Those LAB strains that were able to inhibit pathogens in higher dilutions (either 1:10 or 1:100) can be considered as a potential antimicrobial agent producing candidates.

Two LAB strains (*L. plantarum* 86 and *W. cibaria* 92) showed broad spectrum of inhibitory activities against all six pathogens. The strain *L. plantarum* 86 showed 86%-90% and 20%-46% growth inhibition of MRSA and *E. faecalis* in 1:1 and 1:100 dilutions, respectively. While with respect to Gram-negative pathogens, it showed on an average 80%-87% growth inhibition in the 1:1 dilution but it was having almost negligible inhibition in 1:100 dilution except against *Y. enterocolitica* where it inhibited the growth upto 20%. Similarly, strain *W. cibaria* 92 showed higher antimicrobial activity towards *C. jejuni, S. aureus* and *E. faecalis* in higher dilutions, also. It was seen that different strains of *L. fermentum* possessed discrete spectrum of antimicrobial actions against various pathogens which is in the agreement with the previously reported studies[6]. Similarly, *P. parvulus* AI1 isolate showed broad spectrum of inhibitory activity towards all tested pathogens except *S. aureus* in the fifty percentages diluted (1:1) CFS. In contrast to this, in one of the reported studies[3] pediocin producing strain *P. parvulus* 133 was highly inhibitory to *L. monocytogenes,* although had no effect on other bacteria.

From the results obtained in the microdilution assay in microtiter plate, two isolates *L. plantarum* 86 and *W. cibaria* 92, showing pronounced antimicrobial effect were investigated to characterize the antimicrobial agent. To nullify the effect of organic acids, the CFS obtained by harvesting the actively grown cells were neutralized using 1N NaOH to pH 6.8 and was checked for antimicrobial effect as describe earlier. The neutralized CFS samples were separately treated with two proteolytic enzymes, proteinase K (5 mg/mL) and pronase E (1 mg/mL) to study their effect. The reaction mixtures were then incubated at 37 °C for overnight, followed by heating at 100 °C to inactivate the enzymes and were checked for residual antimicrobial activity. Similarly, neutralized CFS of the LAB isolates was heat treated at 100 °C for 15 min and autoclaved (121 °C /15 min) and subsequently determined for the residual activity as described before.

To precipitate the antimicrobial peptide, supernatant from each isolate was precipitated with 80% ammonium sulphate (Merck, Germany) at 4 °C with gentle stirring for overnight, and centrifuged for 20 min at 14 972 g at 4 °C using Sigma^R 3-18K Labex instrument (AB Helsingborg) to recover the protein precipitates. The brown colour precipitates were dissolved in sodium acetate buffer (pH 5.0) and centrifuged to remove un-dissolved debris. The sample was filled into 1000 D dialysis membrane using sodium acetate buffer (pH 5.0) for two days at 4 °C with intermittent change of buffer. The samples were collected into 15 mL tubes and subjected to measure antimicrobial activity against *E. coli* and *S. aureus* as mentioned earlier.

The antimicrobial agents from both the LAB strains were found to be heat stable, but were almost completely abolished after the treatment with proteolytic enzymes (Figure 2). Heat treatment at 100 °C for 20 min and autoclaving (121 °C /15 min) did not completely destroy the antibacterial activity of the peptide that is in agreement with the other reported studies for *L. plantarum*. Considering the bactericidal activity, proteinaceous nature, heat resistance, and low molecular weight; the antimicrobial peptide can be classified as a small, heat stable peptide presumably belonging to class IIa[1,7]. However, the anti-listerial activity of the isolate is necessary to perform to make it perfectly fit into this definition. Noonpakdee et al.[7] reported that *L. plantarum* PMU33 strain, isolated from *som-fak*, a Thai low salt fermented fish product was producing a bacteriocin that was found to be heat stable at autoclaving temperature (121 °C for 15 min). Similarly *L. plantarum* strain 86 also produced heat stable antimicrobial peptide. It was differing in the antimicrobial spectrum in such a way that it exhibited inhibitory activity against both Gram- positive and Gram-negative bacteria while the previous one showed inhibitory activity against Gram positive pathogens only.

Studies on bacteriocins from *Weissella* spp. remain scarce. Srionnual et al.[8] stated that *W. cibaria* 110, a isolate from Thai fermented fish was able to produce a bacteriocin which showed a narrow spectrum of inhibition. It was not able to inhibit other LAB species, also. The bacteriocin was able to withstand high temperature and catalase treatment, but was sensitive to proteolytic enzymes which are in harmony to present study. In different experiment, *W. cibaria* isolate producing bacteriocin was effective against the indicator strain *Lb. sakei* JCM 1157^T, but not against *L. monocytogenes* ATCC 19111 and *H.*

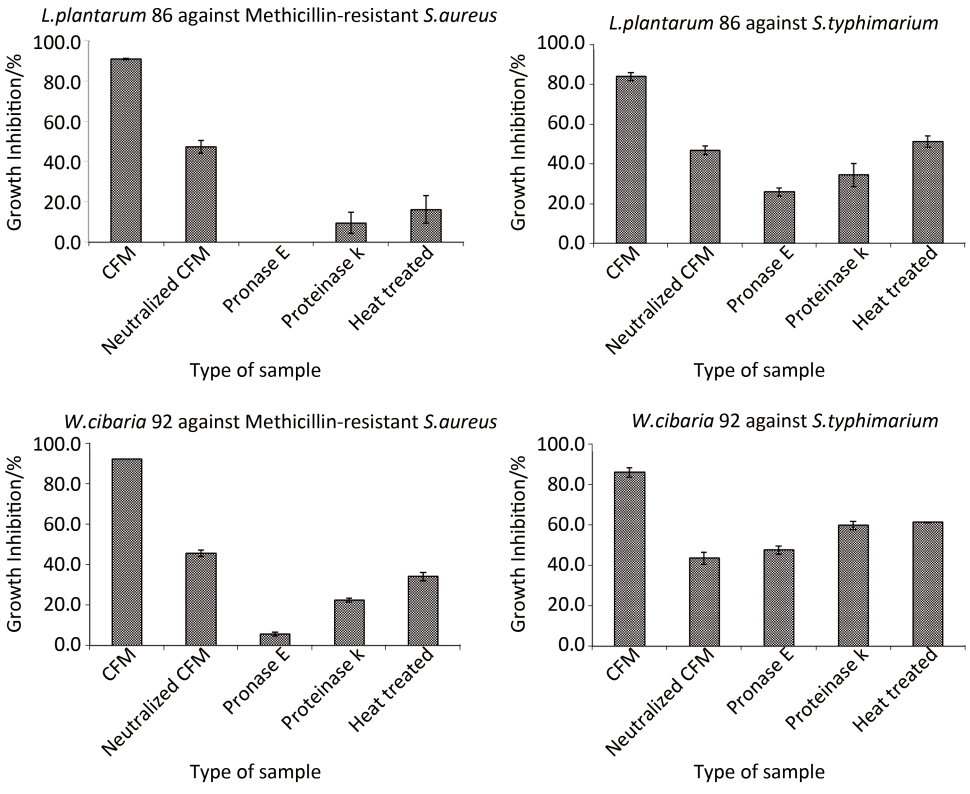

Figure 2. Antibacterial activity from neutralized, enzyme, and heat treated cell free supernatant of LAB strains against pathogens.

pylori ATCC 43504^T. Similarly, bacteriocin produced by *W. cibaria* N23 had a narrow antibacterial spectrum being able to inhibit only *W. confusa* N31[9]. Other studies dealing with different *Weissella* spp. report a broad spectrum of activity towards foodborne/spoilage pathogens including Gram-negative organisms[10]. In agreement to this, in current experiments also strain *W. cibaria* 92 and 142 as well as *W. confusa* AI10 showed higher antimicrobial activity against clinical pathogens such as *C. jejuni, S. Aureus,* and *E. Faecalis.*

In conclusion, current study demonstrates that vegetables and traditional fermented food products offer an alternative and readily available source of LAB strains with antimicrobial activities against clinical pathogens. In the present study, two LAB strains, *L. plantarum* 86 and *W. cibaria* 92 showed broad spectrums of inhibitory activities against all the pathogens tested. Preliminary characterization of the antimicrobial agents revealed that both the strains produce a low molecular weight peptide. The prospective experiments should focus on the characterization of amino acid and nucleotide sequences of these antibacterial compounds.

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