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Potential of bacteriocinogenic *Lactococcus lactis* subsp. *lactis* inhabiting low pH vegetables to produce nisin variants

Sara Sadiq [a, d], Muhammad Imran [a], Muhammad Nadeem Hassan [a], Mazhar Iqbal [b], Yusuf Zafar [c, d], Fauzia Yusuf Hafeez [a, *]

[a] Department of Biosciences, Faculty of Sciences, COMSATS Institute of Information Technology, Park Road, Islamabad, Pakistan
[b] Health Biotechnology Division, National Institute for Biotechnology & Genetic Engineering – NIBGE, Faisalabad, Pakistan
[c] Pakistan Atomic Energy Commission – PAEC, Islamabad, Pakistan
[d] Department of Plant Genomics and Biotechnology, PARC Institute of Advanced Studies in Agriculture, National Agricultural Research Centre, park Road, Islamabad, Pakistan

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ABSTRACT

Antimicrobial behavior of lactic acid bacteria (LAB) has been explored since many years to assess their ability to produce bacteriocin, a natural preservative, to increase the shelf life of food. This study aims to characterize bacteriocin producing strains of lactic acid bacteria isolated from acidic to slightly acidic raw vegetables including tomato, bell pepper and green chili and to investigate their potential to inhibit food related bacteria. Among twenty nine LAB screened for antimicrobial activity, three exhibited antagonism against closely related bacterial isolates which was influenced by varying temperature and pH. They were identified up to strain level as *Lactococcus lactis* subsp. *lactis* TI-4, *L. lactis* subsp. *lactis* CE-2 and *L. lactis* subsp. *lactis* PI-2 based on 16S rRNA gene sequence. Their spectrum of inhibition was observed against food associated strains of *Bacillus subtilis* and *Staphylococcus aureus*. Moreover, *L. lactis* subsp. *lactis* PI-2 selected on the basis of higher antimicrobial activity was further evaluated for bacteriocin production which was detected as nisin A and nisin Z. These findings suggest the possible use of *L. lactis* strains of vegetable origin as protective cultures in slightly acidic as well as slightly alkaline food by the bio-preservative action of bacteriocins.

1. Introduction

Fermentation by lactic acid bacteria (LAB) not only makes the food more palatable but also improves its shelf life, so their use in food processing has been practiced since many years (Sullivan, Ross, & Hill, 2002). They are Generally Regarded as Safe (GRAS) (Settani & Corsetti, 2008) and also serve as probiotics (Aslam & Qazi, 2010). They include genera of *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus* and *Weissella* (Stiles & Holzapfel, 1997).

Food commodities like meat, milk and milk products, beverages, bakery products, fruits and vegetables are the source of LAB. Similarly fermented vegetables like green tomatoes, pepper, cucumbers inhabit different types of LAB (Grosu-Tudor & Zamfir, 2011). Different micro flora prevails in vegetables especially *Lactococcus* (Kelly, Asmundson, & Huang, 1996), depending upon the type of vegetable, environment and handling procedures (Sajur, Saguir, & Manca de Nadra, 2007). Antimicrobial activity of LAB is reported against closely related strains as well as food borne pathogens e.g. *Bacillus cereus* (Bromberg, Moreno, Zaganini, Delboni, & Oliveira, 2004), *Listeria monocytogens*, *Staphylococcus aureus*, *Bacillus subtilis* and spores of *Clostridium perfringens* (Saranya & Hemashenpagam, 2011). They exhibit antimicrobial activity by producing substances like organic acids, hydrogen peroxide, diacetyl compounds and bacteriocins (Klaenhammer, 1988). Bacteriocin, an antimicrobial protein which is active against closely related bacterial species (Parada, Caron, Medeiros, & Soccol, 2007), has gained popularity in recent years to be used as bio preservative to fulfill the consumer demand for natural and less processed food.

Fermented products are the best source of bacteriocin producing LAB (Kawamoto & Shima, 2004) like fermented sausages (Vermeiren, Devlieghere, & Debevere, 2004), fermented fish products (Lee & Paik, 2001) and fermented vegetables (Cheigh et al., 2002). On the other hand unfermented organic leafy vegetables are also the source of bacteriocin producing LAB strains

* Corresponding author. Tel.: +92 300 6602614.
E-mail address: fauzia@comsats.edu.pk (F.Y. Hafeez).

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(Ponce, Moreira, del Valle, & Roura, 2008). LAB can produce more than one bacteriocin (Eijsink et al., 2002). *Lactococcus* spp. such as *Lactococcus garvieae*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* produce different types of bacteriocin (Borrero et al., 2011; Holo, Nilssen, & Nes, 1991; Renata, Moreno, Delboni, & Cintra, 2006;; Settanni & Corsetti, 2008). Nisin is produced by *L. lactis* subsp. *lactis* and is the only bacteriocin which is approved by Food and Drug Administration (FDA) and is being used as bio preservative in more than 50 countries (Delvis-Broughton, 2005). Nisin is a lantibiotic having 34 amino acids (Cheigh et al., 2002) and has different variants including A, F, Z and Q which are produced by *L. lactis* (Piper & Hill, 2011).

Application of bacteriocin producing LAB as protective cultures (Galvez, Abriouel, Lopez, & Omar, 2007) like preservation of yogurt (dahi) by nisin producing strain of *L. lactis* W8 (Mitra, Chakrabartty, & Biswas, 2010) and their bacteriocin are the best replacement of chemical additives and intense physical treatments to preserve food against spoiling bacteria. Research work has been done since several years for the isolation and selection of appropriate LAB for the production of bacteriocin. Moreover, *Lactococci* existing in milk system has been extensively focused (Soomro, Masud, & Anwaar, 2002). However, their existence in raw vegetables under acidic to slightly acidic environment is not yet studied. The present study was executed to reveal the prevalence of bacteriocin producing LAB in tomato, bell pepper and green chili, followed their genetic identification up to strain level. Their antimicrobial behavior was evaluated at different temperature and pH against indicator strains. Moreover, evaluation of bacteriocin activity was further extended against food spoiling and pathogenic bacteria. Finally, the produced bacteriocin was purified and characterized by chromatographic and mass spectrometric techniques.

2. Materials and methods

2.1. Bacterial test and indicator strains, screening and culture conditions

LAB were isolated from internal as well as external parts of different raw vegetables (green chilies, tomatoes and bell peppers). The vegetable samples (1 g) were homogenized and serially diluted up to 10^{-6} in 9 mL of sterile saline solution (0.85 g/mL). Twenty nine LAB were isolated by spreading last two dilutions on de Man, Rogosa, Sharpe (MRS) agar (Oxoid, England-pH 6.2) plates, incubated at 30 °C overnight. Three LAB isolates were selected for further experiment on the basis of their initial antimicrobial activity against each other assayed by spot-on-lawn method (Pingitore, Salvucci, Sesma, & Nader-Macías, 2007) (Table 1). For screening, LAB isolates were incubated in MRS soft agar (0.7 g/mL) at 30 °C for 24 h. The lawn (50 μL) of isolated LAB as indicator strains (to detect the antimicrobial activity of test strains) was prepared on MRS agar plates followed by spotting 5 μL of test strain on the same plate and incubated at 30 °C overnight. The test strains forming clear zones around them were selected. Antimicrobial activity of the selected isolates was also tested against *S. aureus* ATCC 25923, *B. subtilis* EU627167 and *Escherichia coli* O157:H7 (Microbiology Lab Collection, COMSATS, Islamabad). They were confirmed as LAB as they formed clear zones around them when 5 μL of their inoculum previously grown in LB broth was spotted on MRS agar plates containing $CaCO_3$ (Daejung, Korea) (1 g/100 mL MRS agar) (Kopermsub & Yunchalard, 2010). LB broth (100 mL) was prepared by 1 g tryptone (Biomark, India), 1 g NaCl (Riedel-de Haen, Germany) and 0.5 g yeast (Lab M, UK). The potential bacteriocinogenic LAB were maintained at −20 °C in glycerol solution.

Table 1
Inhibitory effect of *Lactococcus lactis* against Gram positive and Gram negative bacteria (The LAB were selected on the basis of their initial activity against closely related indicator strains).

Bacterial strain	Origin	*L. lactis* TI-4	*L. lactis* CE-2	*L. lactis* PI-2	*Bacillus subtilis*	*S. aureus*	*E. coli*
L. lactis PI-2	Green chili	+	−	−	+	+	−
L. lactis TI-4	Tomato	−	+	−	+	+	−
L. lactis CE-2	Bell pepper	+	−	−	+	+	−

+ = inhibition zone, − = no inhibition zone. The inhibitory effect was performed in 4 replicates by spot-on-lawn method.

To prepare the supernatant, the LAB strains were grown in 1000 mL MRS broth comprising of 10 g peptone (Scharlau, Spain), 8 g beef extract (Biomark), 4 g yeast (Lab M), 20 g glucose (Riedel-de Haen), 5 g sodium acetate (Serva, Germany), 1 mL Tween 80 (Sigma Aldrich, Germany), 2 g dipotassium hydrogen phosphate (Serva), 2 g tri-ammonium citrate (Biomark), 0.2 g magnesium sulfate (Scharlau), 0.05 g manganese sulfate (Scharlau). The other indicator strains i.e. *E. coli*, *S. aureus* and *B. subtilis* were cultured in LB broth at 37 °C.

2.2. Molecular characterization of bacteriocin producing LAB

2.2.1. DNA extraction and 16S rRNA gene amplification and sequencing

The genomic DNA of the selected LAB was obtained by CTAB method. The 16S rRNA gene was amplified with the help of P1 (5′-AGAGTTTGATCCTGGTCAGAACGAACGCT-3′) and P6 (3′-TACGGC-TACCTTGTTACGACTTCACCCC-5′) (Wei, Wang, Tan, Zhu, & Chen, 2002). For gene amplification, the thermocycler was operated at 95 °C for 5 min, followed by 25 cycles of 45 s at 95 °C, 45 s at 60 °C, 1 min at 72 °C and 10 min at 72 °C as final extension. A sharp band of 1500 bp was observed when PCR product was passed through agarose gel (1 g/100 mL TAE buffer) stained with ethidium bromide. The freshly amplified product was cloned into the vector PTZ57R/T of Fermentas Cloning Kit #K1214. The cloned gene was sequenced by Macrogen Korea, Seoul, Republic of Korea.

2.2.2. Analysis of sequencing data

The obtained nucleotide sequences and previously published sequences were compared to find out maximum homology by using EzTaxon (Chun et al., 2007).

2.3. Phylogenetic tree construction

For tree construction the sequences were retrieved from EzTaxon (Chun et al., 2007) based on maximum homology with bacteriocinogenic strains. The sequences were multiple aligned by using ClustalW program. Neighbor joining tree was constructed by means of Mega 5.1 software, taking 1000 as bootstrap value.

2.4. Effect of incubation temperature and pH of media on antimicrobial activity of LAB

Different incubation temperature and pH ranges were evaluated for optimum antimicrobial activity by performing spot-on-lawn method as mentioned above. A spot of 5 μL of test strains was applied against the lawn (50 μL) prepared by their respective indicator strains of LAB. The effect of temperature was tested by incubating the prepared plates at 25 °C, 30 °C, 33 °C and 37 °C. To test the influence of concentration of target strains on the inhibition spectrum of test strains, lawn of 50 μL and 100 μL was prepared separately.

To assess the effect of pH, MRS agar was adjusted to pH 5, 7, 8 and 9 by using concentrated HCl or NaOH. The original pH of MRS