Abstract

Lactic acid bacteria (LAB) especially *Lactobacillus plantarum* serve as a functional food having ability to inhibit the growth and attachment of uropathogens to uroepithelial cells to a large extent in vitro. Antibiotic resistance represents a serious global health threat to public health, so the infections, such as pneumonia and urinary tract infections (UTI) are becoming harder to treat. Therefore it is necessary to develop an action plan to restrain the problem of antibiotic resistance. One approach in UTI control could be the use of *Lactobacilli*, due to the fact that these indigenous inhabitants in human intestine have been found to play important role in protecting the host from various infections. This study aimed to isolate and identify LAB from indigenous sources of rotten fruits and vegetables in order to select LAB strains, with remarkable antimicrobial activities against multi-drug-resistant uropathogens and determining probiotic characteristics of *Lactobacillus plantarum* isolates including production of bacteriocin substances and the optimization of the biomass production.

The study on isolation and identification of *lactobacilli* was conducted at The Food and Biotechnology Research Centre, Pakistan Council of Scientific and Industrial Research (PCSIR) Lahore, Pakistan. The comparative study between *Lactobacillus plantarum* species from different regions (Pakistan and Ireland) and different sources *(cucumber,* grapes, apple, wine, strawberry, tomato, cauliflower, brinjal, cheese, cow teats, silage and pickles) was conducted at Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

A total of 185 lactic acid bacteria were isolated from spoiled fruits and vegetables, of which 11 Gram-positive and catalase-negative *lactobacilli* isolates, were identified as *Lactobacillus acidophilus, L. paracasei, L. delbrueckii, L. helvetics and seven strains of L. plantarum.* The latter organism had the highest abundance from majority of the samples, so its taxonomic study was also verified through 16S rRNA gene sequencing. The isolated *lactobacilli* were screened against multi-drug resistant uropathogens: *Candida albricans, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis and Escherichia coli.* Growth inhibition zones (GIZ) were over 10mm against all uropathogenic test organisms, where *L. plantarum* strains demonstrated remarkable

inhibitory activities against *E. coli* and *E. faecalis*, with growth inhibition zones (GIZ) up to 28 mm. The susceptibility test to 16 antibiotics showed multidrug resistance (three to five antibiotics) among all the tested uropathogens. The obtained results revealed that all the *lactobacilli* isolates displayed antimicrobial activity against six out of seven antibiotic resistant uropathogens, indicating that these bacteria could represent a good ecological plan for control and prevention of urinary tract disease.

The results obtained in the comparative study between 14 *Lactobacillus plantarum* strains showed 10 distinctly different *L. plantarum* strains after PFGE patterns. Antimicrobial screening revealed, *L. plantarum* AS-4, AS-6, AS-8, AS-13 and AS-14 strains as the potential producers of bacteriocin as antimicrobial activity of their culture supernatants expressed GIZ up to 12, 12, 14, 11 and 13mm respectively against *L. innocua* (food born pathogen). The positive control, previously characterized plantaracin producer strain LMGP-26358 also showed GIZ of 12mm. The *L. plantarum* DPC 6637, DPC1122, DPC6429 strains showed a narrow spectrum of activity while AS-7, AS-9, DPC2064, DPC5260 along with lactacin producer and nisin producer positive control strains, showed no activity against *L.innocua.* On the other hand all the *L. plantarum strains* were active against a broad range of microorganisms including *L. monocytogenes* DPC 6179, *Enterococcus facelis* 5055 (LMG9737), *E. coli* DPC EC101, *Bacillus subtilis* LMG 8198, *Clostridium perfringens* LMG 10468, *Clostridium difficile* ATCC 42593, *Staphylococcus aureus* DPC 6867. However some *L. plantarum strains* AS-7, AS-9 and DPC 2064 expressed no GIZ against *Clostridium perfringens* LMG 10468, *Clostridium difficile* ATCC 42593. None of the 15 LAB strains and positive control LMGP-26358 displayed the bacteriocin activity against gram positive *Lactobacillus bulgaricus* 081211 while lactacin producer and nisin producer strains were but weakly inhibited. The antimicrobial substance of *L. plantarum* strains AS-4, AS-6, AS-7, AS-13 and AS-14 was purified by HPLC and the molecular mass was determined by using MALDI-TOF mass spectrometry as 3932 Da along with control (*L. plantarum* LMGP-26358).

Molecular characterization of these isolates was done by amplification of previously known bacteriocin genes. Polymerase chain reaction analyses revealed that plantaracin genes were present in the genome of *L. plantarum* strains AS-4, AS-6, AS-7, AS-13 and

AS-14 along with *L. plantarum* LMGP-26358 and almost the similar growth pattern of bacteriocin production was observed by these strains. Single GIZ were observed from plantaracin of *L. plantarum* strains AS-4, AS-6, AS-7, AS-13, AS-14 and control, showing the production of one type of bacteriocin. The production of plantaracin was first detected 4 h after inoculation. Plantaracin production then increased, reaching a maximum of 2560 AU/ml after 12 h. Plantaracin activity remained stable at this level for the next 40 hrs, before declining to a level of 500 AU/ml after 120 h of incubation. The products were highly thermostable, retaining their activity even after treatment with heat for half an hour at 40, 50, 60, 70, 80, 90 and 100°C.

The partially purified plantaracin exhibited stability at 100°C for 30 min, with the exception for *L. plantarum* AS-7 and AS-9 strains. None of the inhibitory zones were affected by catalase, indicating that hydrogen peroxide was not involved in the inhibitory action. In contrast, the antimicrobial activity associated with thirteen out of fifteen strains was found to be sensitive to proteinase K, and pepsin indicating the presence of antimicrobial compound of a protein nature in these cases. The loss of activity confirmed that the antimicrobial substance produced by *L. plantarum* was indeed proteinaceous. The *L. plantarum* strains worked out in this study are promising and further investigation of their technological potential in relation to their use in dairy food products to avoid *Listeria* contamination be focussed. All the stains were stable at pH 3, showing viable counts of 6 log CFU /ml by the end of 4 h as compared to viable counts of 7 log CFU/ml of control strains, whereas on exposure to pH 2 the viability of strains limited, dropped to 2 and 3 log CFU/ml, respectively This showed that the best pH to select for strains with probiotic potential was pH 3. Following 4h exposure to pH 3, eighteen *L. plantarum* strains were able to tolerate and grow in the bile supplemented conditions as three *L. plantarum* strains AS-9, DPC5260, DPC2064 showed a viable count of 5 log CFU/ml after 24h in MRS broth supplemented with 0.5% bile, except only one strain *L. plantarum* DPC4229 which displayed a viable count of 3log CFU/ml. Viable counts increased to reach levels of 5 and 6 log CFU/ml for 11 of the assayed strains after a 24 h period of incubation in MRS supplemented with 0.3% bile. All the *L. plantarum* strains showed a significant increase in viable counts with level of 7-8 log CFU/ml within the 24

h period of exposure to MRS-bile broth supplemented with 0.2% concentration of bile salt. The present results showed that the LAB isolates could have ability to tolerate and grow in broth containing 0.5% bile, while they grew well with 0.3% bile and expressed steady

growth in the presence of 0.2%bile. Similarly, the resistance of 14 *L. plantarum* strains after exposure to simulated gastric juice conditions at pH 2 resulted into non significant difference in viability ($P > 0.05$) of control and test strains.

The strains AS-4, AS-14, DPC6429 and AS-14 showed still higher counts i-e 7 log CFU/ml, and the resistance of strains was observed with population reduction of about 3log CFU/ml for DPC6637, 4log CFU/ml for DPC4229, 5 log CFU/ml for DPC2064 after 2hours. The viable cell numbers of all 14 strains after 24 h exposure to the simulated gastric juice, were reduced from 9log CFU/ml to < 2log CFU/ml as compared to control, indicating that *L. Plantarum* strains can survive during gastric environments (pH 2) after 2 and 3hours. All strains showed weak growth on 0.2% sodium taurocholat-MRS agar plates incubated at 37°C for 72 h, while positive control *L. gasseri* DPC 6840 showed good hydrolytic activity. No activity was observed by any single strain tested against 0.5% sodium taurocholat-MRS agar plates along with positive control.

Not a considerable decrease in viability was detected for the 14 *L. plantarum* as well as the control strains after exposure to different salt concentrations (2.5%, 5% and 7.5% NaCl), whereas none of the strain tolerated 10% NaCl concentration.

Safety investigations revealed that all of the *L. plantarum* strains were sensitive to commonly used antibiotics. Vancomycin and tetracycline did not inhibit the growth of any of the strains tested. The isolates were susceptible to gentamycin, kenamycin, streptomycin, neomycin, ciprofloxacin, clindamycin, chloramphenicol, ampicilin, penicillin, erythromycin; virginiamycin, linzolid, trimethoprim, rifampicin. Screening of *L. plantarum* strains for the conversion of linoleic acid to conjugated linoleic acid along with control strain *Bifidobacterium breve* NCFB 2258 at a wavelength of 233 nm showed 51.75% conversion of linoleic acid to c9, t11 CLA and (0.40 % conversion) to t10, c12 CLA by *B. breve* strain. All of the remaining strains converted linoleic acid to CLA, as AS-4 produced 1.44% c9, t11 CLA, AS-13 produced 4.03% c9, t11 CLA, AS-14

produced 4.23% c9, t11 CLA, Lp-Choc produced 4.23% c9, t11 CLA, whereas strain DPC 6429 did not showed any conversion.

The best optimum medium composition at which biomass of 16.04g/L (DCM), was obtained was: 75 g/L cheese whey, 20 g/L glucose, 8g/L yeast exract, 20g/L corn steep liquor and 20 g/L of ammonium bisulphate and pH 6.2 with temperature 40°C for *L. plantarum* strain AS-14. Comparative studies showed that cultivation using cheese whey as carbon source and corn steep liquor as nitrogen source with other selected optimized medium components generated higher biomass production than commercial MRS medium yielding dry cell mass of 10.78 g/L at pH 6.2 and temperature 40 °C with lower cost.