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Molecular Characterization of Bacteriocinogenic, Antifungal and Probiotic Lactic Acid Bacteria Isolated from Chicken Gastrointestinal Tract

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Abstract

In this study, bacteriocinogenic *Lactobacillus plantarum* isolates capable of inhibiting food- and feed-borne filamentous fungi from the gastrointestinal tract (GIT) of broiler chicken were identified using 16S rRNA gene sequencing and further evaluated for probiotic properties *in vitro*. Six potent lactobacilli were selected from one hundred and thirteen isolates for the present study based on their ability to inhibit both pathogenic bacteria and filamentous fungi. They were characterized using various physiological, biochemical and molecular methods. They were acetoin producers, homo fermentative, catalase-negative and producing racemic lactic acid (10 - 20 mM). All the six isolates exhibited varied sugar utilization and RAPD pattern, indicated their strain level genotypic variation. The 16S rRNA gene sequence and multiplex PCR analysis confirmed that these isolates were *Lactobacillus plantarum.* The isolates being resistant to low pH (2.0) and bile salt (0.6%) could survive in the gastrointestinal tract of host indicating their potential probiotic application. The isolates were non-pathogenic (*γ*-hemolytic) and exhibited resistance to antibiotics ciprofloxacin, nalidixic acid, norfloxacin, nitrofurantoin, colistin and streptomycin. They demonstrated strong autoaggregating phenotype ranging from 78% to 86% and showed 49% - 61% and 30% - 46% coaggregation with *E. coli* MTCC 728 and *L. monocytogenes* MTCC 657, respectively. The percentage of hydrophobicity ranged from 16% - 33% for all the isolates showing that surface was rather hydrophilic. They exhibited *β*-galactosidase activity ranging from 1036 - 1179 MU, bile salt hydrolase activity assisting to reduce serum cholesterol and produced the anti-*Listerial* bacteri-

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ocin. The strong inhibitory activity of these isolates against food spoilage molds and bacteria with probiotic properties indicates their potential application as food preservatives.

Keywords

Broiler Chicken, Filamentous Fungi, *Lactobacillus plantarum*, Probiotic

1. Introduction

Molds are frequent and important spoilage organisms in the food and feed systems, causing huge economic losses to industrials and health hazards to consumers [1]. The major species involved in the food spoilage are *Aspergillus*, *Fusarium*, *Penicillium* and *Mucor*. Chemical preservatives such as propionic, sorbic and acetic acids and their salts only decrease fungal infections and fall short of contaminant elimination. Additionally, some molds have acquired the ability to degrade chemical preservatives such as sorbate [2]. The raising consumer concerns over the usage of chemical preservatives in food processing [3] and their focus on minimally processed green-labeled foods is driving the food industry towards lactic acid bacterium based food preservation.

Biopreservation is the use of lactic acid bacteria (LAB) and/or their antimicrobial compounds to prevent spoilage and to extend the shelf life of foods. LAB has a long history of use as a bio-preservative and is "generally regarded as safe" (GRAS). LAB is Gram-positive, anaerobic but aero-tolerant, non-spore forming, non-motile, rod and coccus-shaped organisms. The preserving ability of LAB is mainly related to the production of organic acids and reduction of pH, which generally restricts growth of pathogenic bacteria and fungi [4]. However, there are other metabolic products such as cyclic dipeptides and bacteriocins/antimicrobial peptides reported to involve in the preservation of many processed and natural foods [5]. There are many reports on antifungal activities and possible application as biopreservatives of LAB from various sources [6] [7].

The GIT of chicken harbors a huge collection of micro flora more than 650 species, of which more than half are previously unknown bacterial genera [8]. Lactobacilli from chicken origin are a good source of antimicrobial peptides [9]. A very limited data are available in the literature on antibacterial and antifungal probiotic lactobacilli isolated from chicken GIT, which is known for harbouring highly diversified microbial species [10]. Probiotics are defined as live microorganisms that when administered in adequate amounts, they confer a health benefit on the host [11]. The principle requisite for selection of good probiotic includes product safety for human and animal consumption and survival in the GIT.

In the present study, we have isolated and characterized LAB from chicken GIT with strong and broad antibacterial and antifungal activity against pathogenic food spoilage microbes. The probiotic characteristics were also tested *in vitro*.

2. Materials and Methods

2.1. Isolates, Cultures and Growth Conditions

LAB was isolated from the crop, intestine, gizzard and ceca of seven weeks old broiler chicken. Serially diluted samples were inoculated onto acidified de Mann-Rogosa-Sharpe (MRS) agar (Himedia, India) and incubated anaerobically at 37˚C for 48 h. The MRS agar plates were acidified at pH 4.0 and supplemented with 0.2% (W/V) sodium azide to support the growth of LAB during isolation. After cultivation, creamy, opaque and elevated single colonies were picked and streaked on MRS agar. Gram positive, catalase negative isolates were selected and stored at –70˚C in MRS broth with 30% glycerol.

The molds *Aspergillus niger* MTCC 4325, *Eurotium species* MTCC 4647, *Penicillium expansum* MTCC 8241, *Penicillium roqueforti* MTCC 933, bacterial pathogens *Listeria monocytogenes* MTCC 657, *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 728, *Aeromonas hydrophila* MTCC 1739, *Pseudomonas aeruginosa* MTCC 2295 and LAB *Lactobacillus plantarum* MTCC 6161, *Lactobacillus lactis* MTCC 3038, *Lactobacillus fermentum* MTCC 1745, *Leuconostoc mesenteroides* MTCC 107, *Lactococcus lactis* subsp. *Chacetylactis* MTCC 3042, *Lactobacillus rhamnosus* MTCC 1408 were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The target fungi were selected as representative of baked goods spoilage fungi and bacterial pathogens were selected representing frequent food contaminants.

Molds were cultivated on potato dextrose agar (PDA) (Himedia, India) slants at 30˚C for 6 days or till sporulation and stored at 4˚C. The spores were harvested from slants in sterile peptone water (0.2% W/V) to prepare the inoculum containing 1×10^5 spores per mL. Bacterial pathogens were cultured in tryptone-glucose-yeast extract (TGY) (Himedia, India) broth at 37˚C for 24 h and stored at –70˚C in TGY broth with 30% glycerol. *E. coli* DH5*α* was grown in Luria-Bertani (LB) medium at 37˚C and 180 rpm. *E. coli* transformants were cultivated in LB medium supplemented with ampicillin (50 µg/ml) (Hi media, India). Plasmid pTZ57R/T (Thermo Scientific, USA) was used for the construction of recombinant plasmid carrying the plantaricin EF gene.

2.2. Primary Screening of Isolates for Antifungal Activity

Bacterial isolates grown in 5 mL of MRS broth at 37˚C for 24 h were checked for their antifungal activity against target fungi by the overlay method described by Y.I. Hassan and L.B. Bullerman, 2008 [12] with minor modifications. A total of 10 µL of each LAB isolate were added as a discrete spots in the centre of buffered MRS agar plates and incubated anaerobically at 37˚C for 24 h. The medium was buffered at pH 6.5 by 75 $m \cdot mol \cdot L^{-1}$ KH_2PO_4 to avoid the effect of pH on antifungal activity. After incubation, the plates were overlaid with 15 mL soft PDA (0.8% agar) containing 1×10^5 spores per mL and incubated at 30˚C. The incubation period lasted up to 4 days and clear zones of inhibition around the bacterial spot were recorded and scored as follows: (−) no visible inhibition; (+) spore formation delayed with a small clear zone around the colony; (++) spore formation delayed with a good clear zone around the colony; (+++) extensive suppression of spore formation and mycelial growth with definite clear zone around the colony. The experiment was performed in triplicates and *Lactobacillus* isolates with very good antagonistic activity were selected for further studies.

Antifungal activity of LAB isolates on the growth of *Aspergillus niger* was investigated by dual agar plate assay or confrontation assay described by Y.I. Hassan and L.B. Bullerman, 2008 [12] with minor modification of using buffered MRS agar. MRS agar buffered at pH 6.5 was mixed with 100 µL of actively grown LAB culture at the bottom of a petri plate and incubated at 37˚C for 24 h. After incubation, the plates were overlaid with soft PDA and 10 µL of mold spore suspension was inoculated as a discrete spot on to the centre of each plate. Then the plates were incubated at 30˚C for 10 days. The diameters of the growing mold colonies were measured in control and test plates. These data were used to plot growth curves and to select potent antifungal LAB isolate. The nature of antifungal activity was investigated with 10-fold-concentrated culture filtrate treated with acid protease ($2\ mg \cdot ml^{-1}$) (Sigma, India) in 10 mM citrate buffer pH 3.0 and incubated at 37˚C for 3 h (Magnusson and Schnürer, 2001). Before evaluating the antifungal activity, the pH of the supernatant was readjusted to pH value 5.0. Both 10-fold-concentrated MRS broth treated with enzyme and pH adjusted 10-fold-concentrated samples served as controls.

2.3. Screening of Isolates for Antibacterial Activity

Antibacterial activities of LAB isolates were determined against target bacterial pathogens by agar well diffusion method as described earlier [13]. The bacteriocinogenic nature of antibacterial activity was determined by using 10-fold-concentrated CFS treated with acid protease ($2\ mg \cdot ml^{-1}$) (Sigma, India) in 10 mM citrate buffer pH 3.0 and incubated at 37˚C for 3 h. Antibacterial activity was checked at pH 6.0 against *Staphylococcus aureus* and compared with control which was also processed in a similar way without enzyme [14].

2.4. Identification of Isolates

2.4.1. Classical Characterization of Potent Antifungal and Antibacterial Isolates

Physiological and biochemical attributes of potent antifungal and antibacterial LAB isolates were determined by methods as described earlier [15]. Sugar utilization profile of isolates was resolved by using the HiCarbo Kit (Himedia, India). The configurations of lactic acid enantiomers and their concentrations were determined by enzymatic method as described earlier [16] [17].

$$\text{Lactate}(\text{mM}) = \frac{\Delta A_{340} \times 3}{6.22 \times \text{vol of sample} \times 1}$$

where 3 is reaction volume, 6.22 is mM extinction coefficient of NADH at 340 nm and 1 is path length of cuvette in cm.

2.4.2. Antibiotic Susceptibility

Antibiotic susceptibility of isolates was assayed by the disc diffusion method. To determine antibiotic susceptibility, isolate was seeded in MRS agar (1% agar w/v) with 10^8 CFU/mL and allowed to get solidified. The commercial antibiotic discs of different concentrations were placed on the surface of the medium and incubated at 37˚C for 24 h. The resistance and sensitivity were noted as per CLSI/NCCLS standard [18].

2.4.3. Molecular Characterization of Isolates

Genomic DNA was extracted using the method of Anderson and Mckay [19]. RAPD finger printing was performed using the primers M13 (5'-GAGGGTGGCGGTTCT-3') and R2 (5'-GGCGACCACTAG-3') as described earlier [13]. The polymorphic DNA pattern was analyzed by running amplified products in 1.5% agarose gel electrophoresis with 500 bp DNA ladder (Sigma, USA). M13 RAPD profiles were visualized under UV light and analyzed with Quantity one software (Bio-Rad, USA). The similarities were calculated using the Dice coefficient. The dendrogram was constructed by means of the unweighted pair group method using arithmetic averages (UPGMA).

The potent isolates were identified by 16S ribosomal RNA (rRNA) sequencing after amplification using primers fKJ (5'-CATTGGGACTGAGACACTGC-3') and rKJ (5'-CACCGCGACATGCTGATTC-3') [17]. The sequences were compared with reference sequences in Gen Bank at the National Centre for Biotechnology Information (NCBI) using a BLASTn program. Phylogenetic tree for isolates was constructed using the neighbor-joining method with MEGA 5.0 software. The 16S rRNA sequences of isolates were deposited in Gen Bank.

2.4.4. Multiplex PCR

Multiplex PCR assay was performed to differentiate *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* with primers paraF (5'-GTCACAGGCATTACGAAAAC-3'), pentF (5'-CAGTGGC GCGGTTGATATC-3'), planF (5'-CCGTTTATGCGGAACACCTA-3') and pREV (5'-TCGGGATTACCAAA CATCAC-3') [20].

2.5. PCR Amplification and Cloning of Plantaricin EF Gene

PCR amplification of plantaricin gene EF (plnEF) was performed from genomic DNA of isolates using primers plnEF-F (5'-GGTGGTTTTAATCGGGGCGG-3') and plnEF-R (5'-ACTTGATGGCTTGAACTATCC-3') [21]. 50 µL of PCR mixture contained 0.5 µM of primer, 100 ng of genomic DNA, 1 X Taq DNA polymerase buffer, 1 U of Taq DNA polymerase, 0.2 mM of each dNTP and 1.5 mM $MgCl_2$. PlnEF gene amplification was performed in a thermo cycler at 94˚C for 3 min, followed by 29 cycles of 1 min at 94˚C, 1 min at 58˚C and 2 min at 72˚C with an extension of 72˚C for 7 min. Amplified plantaricin EF gene was purified using a clean-up kit (Merck Bioscience, India) and ligated into pTZ57R/T cloning vector by TA cloning method. Ligation mixture was transformed into *E. coli* DH5*α* competent cells, using standard procedure described by Sambrook *et al.*, 2001 [22]. The recombinant plasmid was confirmed by restriction digestion by *EcoR* I and *Hind* III, as well as by sequencing. Sequencing was performed at Eurofins Genomics India Pvt Ltd, India and sequence was submitted to Gen Bank data base.

2.6. Probiotic Properties of Isolates

2.6.1. Auto-Aggregation, Co-Aggregation and Cell Surface Hydrophobicity

Aggregation between the cells of same strains (auto-aggregation) or between genetically different strains (co-aggregation) is of considerable importance in the human gut where probiotics are to be active and such abilities favour bacterial maintenance in the gastrointestinal tract. The autoaggregation and coaggregation (with *E. coli*, *Listeria monocytogenes*) assays were performed according to Osmanagaoglu *et al.*, 2010 [23]. Isolates were grown in MRS broth for 18 h at 37˚C. Cells were harvested and washed twice with phosphate buffer saline (PBS) pH 7.0. The cells were suspended in PBS, pH 7.0 and adjusted to an optical density at A_{600} to1.0. To three milliliters of cell suspension, equal volumes of n-hexadecane was added, vortexed for 2 min and allowed to stand for 30 min for phase separation. The aqueous phase was collected and absorbance was checked at 600 nm. The optical density readings were used to calculate the percentage of cell surface hydrophobicity of isolate adhering to solvent [23].

2.6.2. Growth at Different pH Values and Bile Concentrations

Isolates were grown in MRS broth adjusted to pH 2, 2.5, 3.5, 7.5 and 8.5 for 24 h at 37˚C. The assay was conducted in sterile flat-bottom 96-well microtitre plates. Each well was filled with 180 µl of the medium and inoculated with 20 µl of the overnight cultures obtained in MRS broth (O.D. at 600 nm is 0.2) at 37˚C. Optical density readings were recorded at 600 nm every hour for 8 h and after 24 h, while cultures grown in MRS broth pH 6.5 served as the control [24]. The experiment was performed in triplicates. Similarly, isolates were grown in MRS broth containing 0.05%, 0.1%, 0.3% and 0.6% of oxgall for 24 h. Optical density readings were recorded at 600 nm every hour for 8 h and after 24 h. Isolates grown in MRS broth without oxgall served as control.

2.6.3. β-Galactosidase Activity

Isolates were grown in MRS-lac broth and cells were harvested by centrifugation at 8000 rpm for 10 min. The cells were washed with saline and suspended in the Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 50 mM β-mercaptoethanol pH7.0) and processed as described by Vinderola and Reinheimer, 2003 [25]. The suspended cells were permeabilized with toluene/acetone (1:9, v/v). The reaction mixture having 900 µl of Z buffer, 100 µl of permeabilized cells and 200 µl of ONPG (4 mg/mL) was incubated at 37˚C for 15 min. The reaction was stopped with the addition of 1% sodium carbonate and absorbance was measured at 550 nm and 420 nm. The enzymatic activity was measured in Miller units.

$$\beta\text{-galactosidase activity}\left(\text{MU}\right) = 1000 \times \frac{A_{420} - 1.75 \times A_{560}}{\text{Time of incubation} \times \text{Volume of culture} \times A_{550}}$$

2.6.4. Hemolytic and Bile Salt Hydrolase Activity

For testing hemolytic activity, isolates were streaked on Columbia agar plates (Himedia, India), containing 5% (w/v) human blood, and incubated for 24 h at 37˚C. Blood agar plates were checked for signs of β-hemolysis (clear zones around the colonies), α-hemolysis (green-hued zones around the colonies) or γ-hemolysis (no zones around the colonies) [26]. *Escherichia coli* MTCC 728 and *Streptococcus pyogenes* MTCC 442 were used as control for α- and β-hemolysis, respectively.

Bile salt hydrolase (BSH) activity was assayed according to Lee *et al*., 2011 [27]. Isolates were streaked on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid and 0.37 g $CaCl_2$/L. The BSH activity was semi-quantified by the precipitation zones around the colonies after incubating anaerobically at 37˚C for 24 h.

3. Results and Discussion

3.1. Isolation and Screening of *Lactobacillus* for Antibacterial and Antifungal Activity

Lactic acid bacteria were isolated from GIT of seven weeks old broiler chicken. A total of 113 *Lactobacillus* strains (47 from crop, 8 from gizzard, 27 from intestine and 31 from ceca) were isolated based on their ability to grow on acidified MRS agar medium. All 113 isolates were screened for antibacterial activity against various LAB and food borne pathogens. All isolates showed activity against tested LAB and bacterial pathogens. Approximately 53% (60 isolates) of the total isolates showed good antibacterial activity against bacterial pathogens *L. monocytogenes*, *S. aureus* and *E. coli*, indicating that *Lactobacillus* isolates from chicken origin are potential candidates for producing antibacterial metabolites. These isolates were further screened for antifungal activity against filamentous fungi *Aspergillus niger* and *Penicillium expansum*. Among the tested spoilage fungi, *Aspergillus niger* growth was inhibited by a dominant number of isolates, whereas *Penicillium expansum* growth was affected by few isolates. A total of six bacilli isolates VJC6, VJC11, VJC13, VJC23, VJI16 and VJI17 having similar morphology (**Figure 1**) were chosen for further study, based on their capability to inhibit all tested bacterial pathogens and food spoilage fungi (**Figure 2** & **Figure 3**, **Table 1** & **Table 2**). All the six isolates were found to be bacteriocinogenic in nature as they lost antibacterial activity upon treatment with acid protease [14] [28] (**Figure 2(a)**, while the antifungal activity persisted indicating a non-proteinaceous substance was involved (**Figure 3(b)**). In the earlier study by Y.I Hassan and L.B. Bullerman, 2008 [12], it was reported that *Aspergillus* species were the most resistant to antifungal activity and difficult to inhibit by LAB. In our study, all the six isolates showed complete inhibition of *Aspergillus niger* for 7 days and continued to show same even for 3 months, indicated their preserving ability against fungal spoilage (**Figure S1**). These isolates were identified by 16S rRNA

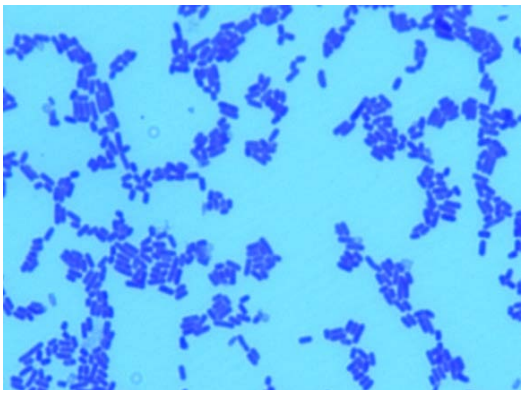
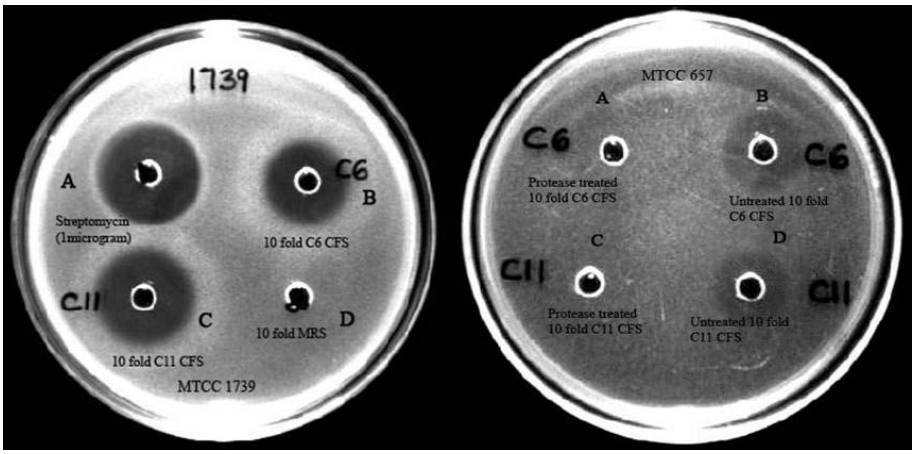
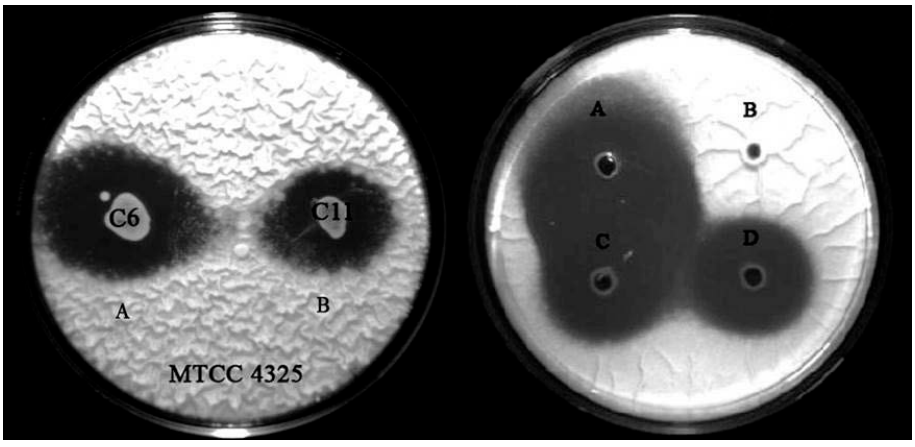


Figure 1. Morphology of one of the bacilli isolates (Gram's staining) from chicken gastro intestinal tract.



(a) (b)

Figure 2. (a) Inhibitory activity against *Staphylococcus aureus*. (A) Streptomycin (1 µg), (B) 10-fold-concentrate of cell free supernatant of VJC6, (C) 10-fold-concentrate of cell free supernatant of VJC13, (D)10-fold-concentrate of MRS broth; (b) Effect of acid Protease on antilisterial activity of 10-fold-concentrated CFS. (A) and (C) are Protease treated 10-fold cell free supernatant of VJC6 and VJC11 respectively whereas (B) and (D) are protease untreated.



(a) (b)

Figure 3. (a) Clear zones of inhibition of growth of *Aspergillus niger* formed around (A) VJC6 and (B) VJC11on MRS agar; (b) Effect of acid protease on antifungal activity against *A.niger* of a 10-fold-concentrated culture filtrate of VJC6. (A) Sodium benzoate (10 mg), (B) 10-fold-concentrate of MRS broth treated with enzyme, (C) and (D) are Protease treated and untreated 10-fold-culture filtrate of VJC6 respectively.

Table 1. Antimicrobial activity against LAB and various pathogens.

Indicator strains	Inhibition zone in mm						
	VJC6	VJC11	VJC13	VJC23	VJI16	VJI17	Positive control
Lactobacillus plantarum (MTCC 6161)	10 ± 0	11 ± 1	10 ± 0	11 ± 1	10 ± 0	10 ± 0	25[a] ± 2
Lactobacillus fermentum (MTCC 1745)	12 ± 1	12 ± 1	11 ± 1	13 ± 1	12 ± 11	12 ± 2	26[a] ± 1
Lactococcus lactis subsp. *lactis* (MTCC 440)	12 ± 2	15 ± 1	12 ± 1	15 ± 2	13 ± 1	12 ± 1	27[a] ± 2
Leuconostoc mesenteroides subsp. *mesenteroides* (MTCC 107)	10 ± 0	11 ± 1	13 ± 1	12 ± 1	13 ± 2	12 ± 1	28[a] ± 3
Lactococcus lactis subsp. *chacetylactis* (MTCC 3042)	12 ± 1	13 ± 1	12 ± 1	14 ± 2	12 ± 1	11 ± 1	28[a] ± 2
Lactobacillus rhamnosus (MTCC 1408)	11 ± 1	11 ± 0	10 ± 0	10 ± 0	10 ± 0	10 ± 0	26[a] ± 2
Pseudomonas aeruginosa (MTCC 2295)	19 ± 1	21 ± 1	20 ± 2	19 ± 1	20 ± 2	20 ± 1	21[b] ± 1
Listeria monocytogenes (MTCC 657)	18 ± 1	21 ± 2	16 ± 1	20 ± 2	16 ± 1	17 ± 2	28[a] ± 3
Staphylococcus aureus subsp. *aureus* (MTCC 737)	21 ± 1	23 ± 2	21 ± 1	20 ± 0	19 ± 1	22 ± 1	26[a] ± 1
Escherichia coli (MTCC 728)	20 ± 1	23 ± 1	19 ± 1	20 ± 0	18 ± 1	19 ± 2	32[b] ± 1
Aeromonas hydrophila subsp. *hydrophila* (MTCC 1739)	18 ± 1	20 ± 0	17 ± 1	20 ± 2	17 ± 1	16 ± 1	23[b] ± 2

[a]Ampicillin (5 µg), [b]Streptomycin (2.5 µg). Mean (± standard deviation) of results of three experiments.

Table 2. Antifungal inhibition spectrum of *Lactobacillus* isolates in agar over layer method.

Indicator organisms	Antifungal performance of isolates					
	VJC6	VJC11	VJC13	VJC23	VJI16	VJI17
Molds						
Aspergillus niger MTCC 4325	++	++	+++	++	+++	++
Aspergillus flavus MTCC	++	++	++	++	++	++
Penicillium expansum MTCC 8241	++	++	++	+	++	++
Penicillium roqueforti MTCC 933	+	+	+	+	+	+
Eurotium species MTCC 4647	++	+	++	+	+++	+
Yeasts						
Candida albicans MTCC	++	+	++	+	++	+
Endomyces fibuliger MTCC 1027	+	–	+	+	++	–

The following scale was used: (–) no visible inhibition; (+) spore formation delayed with a small clear zone around the colony; (++) spore formation delayed with a good clear zone around the colony; (+++) extensive suppression of spore formation and mycelial growth with definite clear zone around the colony.

gene sequence and multiplex PCR analysis. The genomic variability of six isolates was studied by RAPD PCR analysis.

3.2. Biochemical and Physiological Characterization

All the isolates were Gram-positive, catalase-negative, rod shaped, aerobic or micro-aerobic, homo fermentative bacteria, and produce D and L-lactic acid (10 - 20 mM). These morphological characters suggested that all the isolates belonged to the genus *Lactobacillus* [29]. All the *Lactobacillus* strains grew in wide ranges of NaCl concentrations (1% - 6.5%), temperatures (15˚C - 37˚C) and pH (4.5 - 8.5), with optimum growth at pH 6.5. The biochemical tests revealed that all *Lactobacillus* strains were acetoin producers and unable to hydrolyze gelatin, starch and arginine. The carbon utilization pattern was checked for six *Lactobacillus* strains and they can ferment glucose, fructose, sucrose, mannose, lactose and trehalose, but not xylose, raffinose, glycerol, melibiose, sodium gluconate, erythritol, α-methyl-D-glucoside and α-methyl-D-mannoside. The sugar profiles of the six isolates were varied and broad (**Table S1**). Biochemical studies indicated that all the six isolates belonged to *Lactobacillus* genus.

3.3. Antibiotic Susceptibility

All the six isolates were assayed for their susceptibility to 22 antibiotics (**Table S2**). They were found resistant

to inhibitors of nucleic acid synthesis (norfloxacin, nalidixic acid and ciprofloxacin), inhibitors of protein synthesis (amikacin and streptomycin), inhibitors of cell wall synthesis (cepharadine and cephaloridine), an inhibitor of cytoplasmic membrane function (colistin), urinary tract antiseptic (nitrofurantoin) and sensitive to chloramphenicol, tetracycline, erythromycin, lincomycin, mecillinam, ampicillin, penicillin-G, and co-trimoxazole. The observed antibiotic resistance and sensitivity are in accordance with the findings of Mathur and Singh, 1995 [30].

3.4. Genotypic Characterization by RAPD-PCR

To exclude the clonal relatedness among the six isolates, RAPD analysis was performed using two primers M13 and R2. The reproducibility of the RAPD analysis was assessed by comparing the PCR products obtained from two separate cultures of the same strain for all the six isolates. Among two primers, M13 was the most useful for distinguishing six *Lactobacillus* strains, producing a variant DNA banding pattern for all the six isolates (**Figure S2**). The UPGMA dendrogram analysis revealed that the six *Lactobacillus* strains were genetically different at strain level and there was a more genetic distance at the strain level between VJI17, VJC23 and the rest of the isolates (**Figure S3**).

3.5. Comparative 16S rRNA Sequence Analysis and Multiplex PCR Assay

The 16S rRNA gene of the six isolates was sequenced and analysed for similarities using NCBI BLAST search program. It showed that all the six isolates had high sequence similarity (100%) to *Lactobacillus plantarum* (AF1), *Lactobacillus paraplantarum* (N957) and *Lactobacillus pentosus* (MH53). As these three species are genotypically closely related (>97%), 16S ribosomal DNA sequences of the isolates are not suitable for species identification. The phylogenetic tree was constructed using the neighbor-joining method (**Figure 4**). The presumed six *Lactobacillus* strains were further confirmed at the species level by multiplex PCR. Multiplex PCR assay based on recA gene gave the amplification product of 318 bp for all the six isolates belonging to *Lactobacillus plantarum* (**Figure S4**). The 16S rRNA analysis and multiplex PCR assay conferred that these isolates were belonged to *Lactobacillus plantarum*.

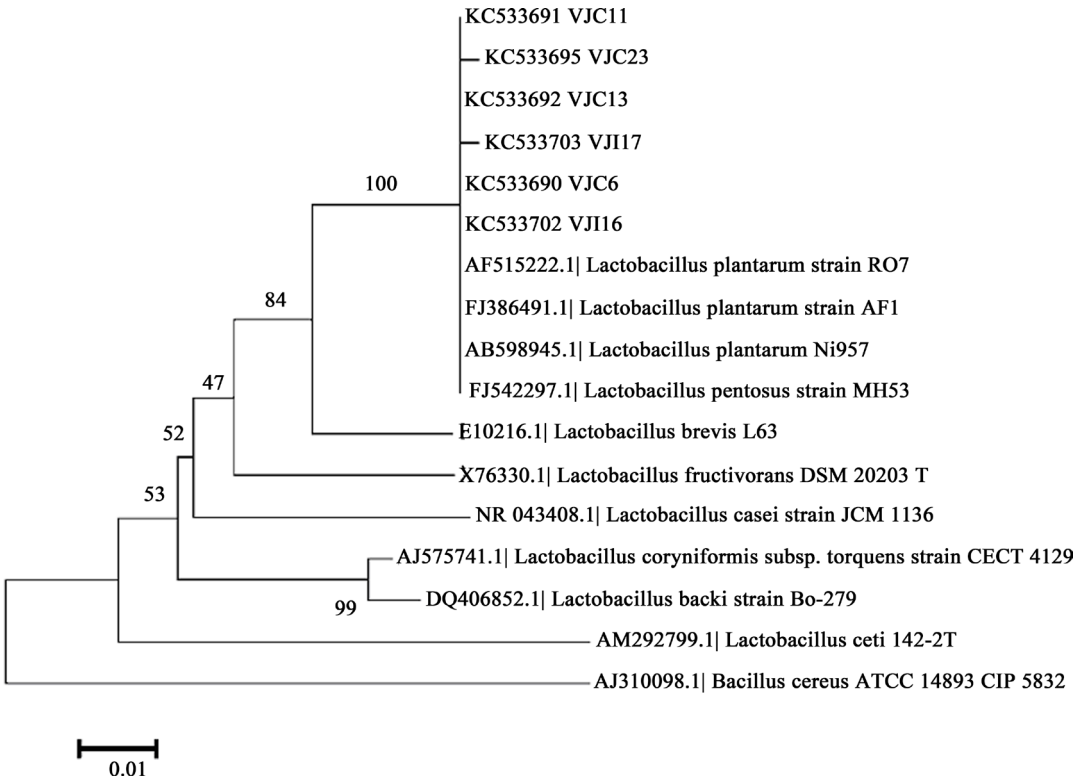


Figure 4. The phylogenetic tree of isolates VJC6, VJC11, VJC13, VJC23, VJI16 and VJI17 constructed by neighbour-joining method.

3.6. Nucleotide Sequence Accession Numbers

The 16S rRNA sequences of *L. plantarum* were deposited in the Gen Bank sequence database under accession numbers KC533690 (VJC6), KC533691 (VJC11), KC533692 (VJC13), KC533695 (VJC23), KC533702 (VJI16) and KC533703 (VJI17).

3.7. PCR Amplification and Cloning of Plantaricin EF Gene

The isolates were tested for the presence of known bacteriocin genes by PCR. Plantaricin EF specific primers plnEF-F and plnEF-R were used to amplify and sequence a 306 bp fragment from all the isolates (**Figure S5**). The nucleotide sequence showed 99% similarity to the plantaricin E and F genes on the genome of *Lactobacillus plantarum* strain C11 (Gen Bank: X94434.2). The sequence analysis by BLAST at NCBI with published sequences revealed that PCR amplified 306 bp fragment consisting of a partial plnE gene (1 - 108 bp) and a complete plnF gene (133 - 291) (Gen Bank: KM347970). The plnEF gene was cloned in to pTZ57R/T cloning vector by TA cloning method and transformed in to *E. coli* DH5α cells. Double digestion of the recombinant vector with *EcoR* I and *Hind* III enzymes, resulted in the release of 400 bp insert and vector fragment (**Figure S6**). Plantaricin is a type IIb two-peptide bacteriocin obtained from different strains of *Lactobacillus plantarum* [31].

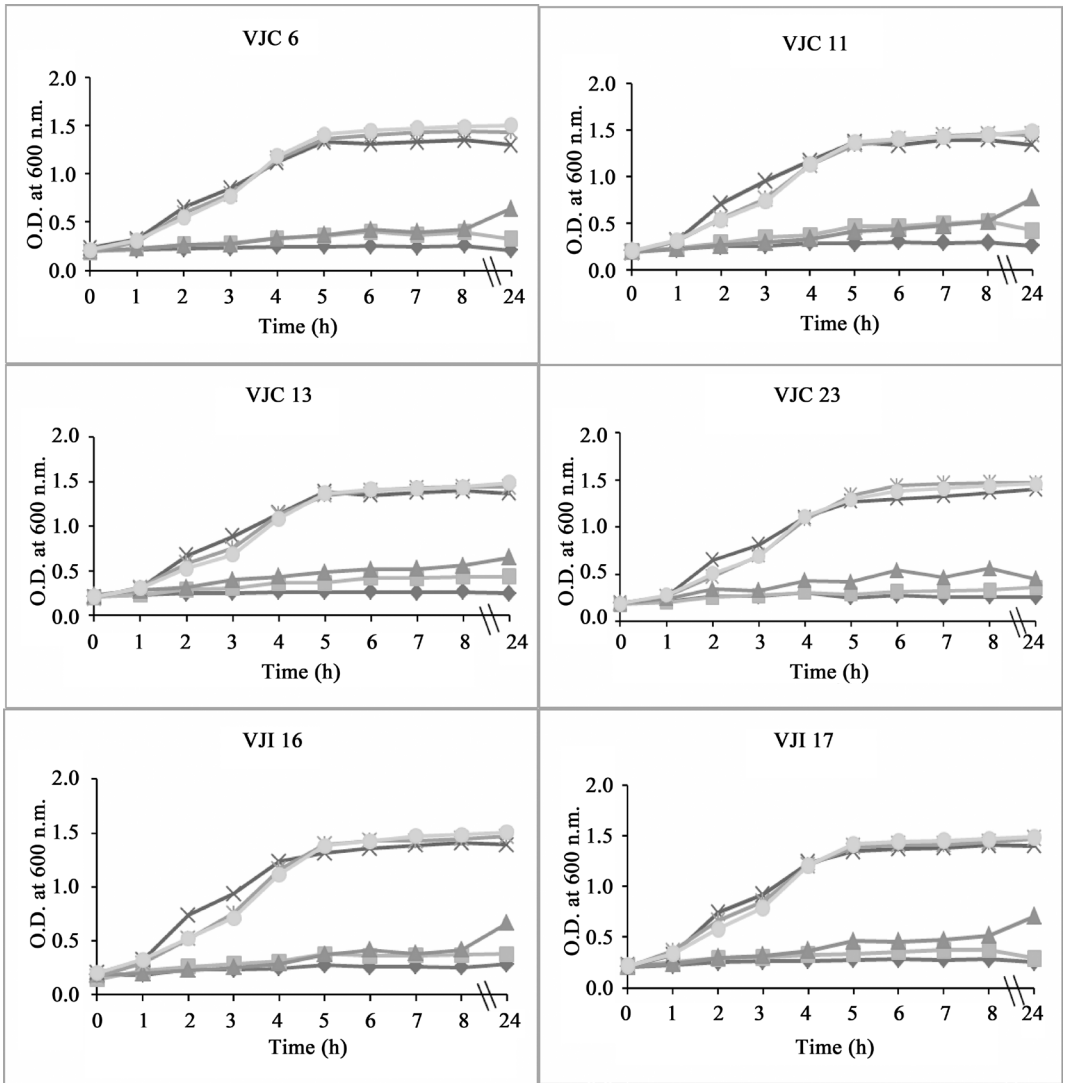


Figure 5. Comparison of growth of isolates VJC6 (A), VJC11 (B), VJC13(C), VJC23 (D), VJI16 (E) and VJI17 (F) in MRS broth at different pH levels.

These findings further confirmed that the isolates were belonged to *Lactobacillus plantarum* and bacteriocinogenic nature may be due to the expression of plnE and F genes.

3.8. Probiotic Properties of *Lactobacillus*

Autoaggregation and coaggregation are vital properties of a probiotic organism, responsible for biofilm formation on the gut epithelium. The binding of probiotic organism with intestinal pathogens will be helpful to kill pathogens by antimicrobial substances [32]. All the isolates exhibited bacteriocinogenic mode antibacterial activity. The tested isolates exhibited strong autoaggregating phenotype ranged from 78% - 86%, which assist in binding firmly with intestinal epithelium. The *Lactobacillus* strains showed the most coaggregation ability with *E.coli* MTCC 728 (49% - 61%) when compared to *L. monocytogenes* MTCC 657 (30% - 46%). The resistance to harsh conditions of the stomach and intestine is another essential prerequisite for probiotic organism. The physiological pH of the stomach is 2 - 3 and bile concentration is 0.3% [33]. A good probiotic must be resistant to the acidic nature of the stomach and the physiological bile concentration. The isolates were remained unaffected by the acidic pH 2.0 - 3.5 and good growth was observed at pH 6.5 - 8.5 (**Figure 5**). They also showed resistance and growth in the presence of 0.6% of bile, indicating that these isolates can survive even at high

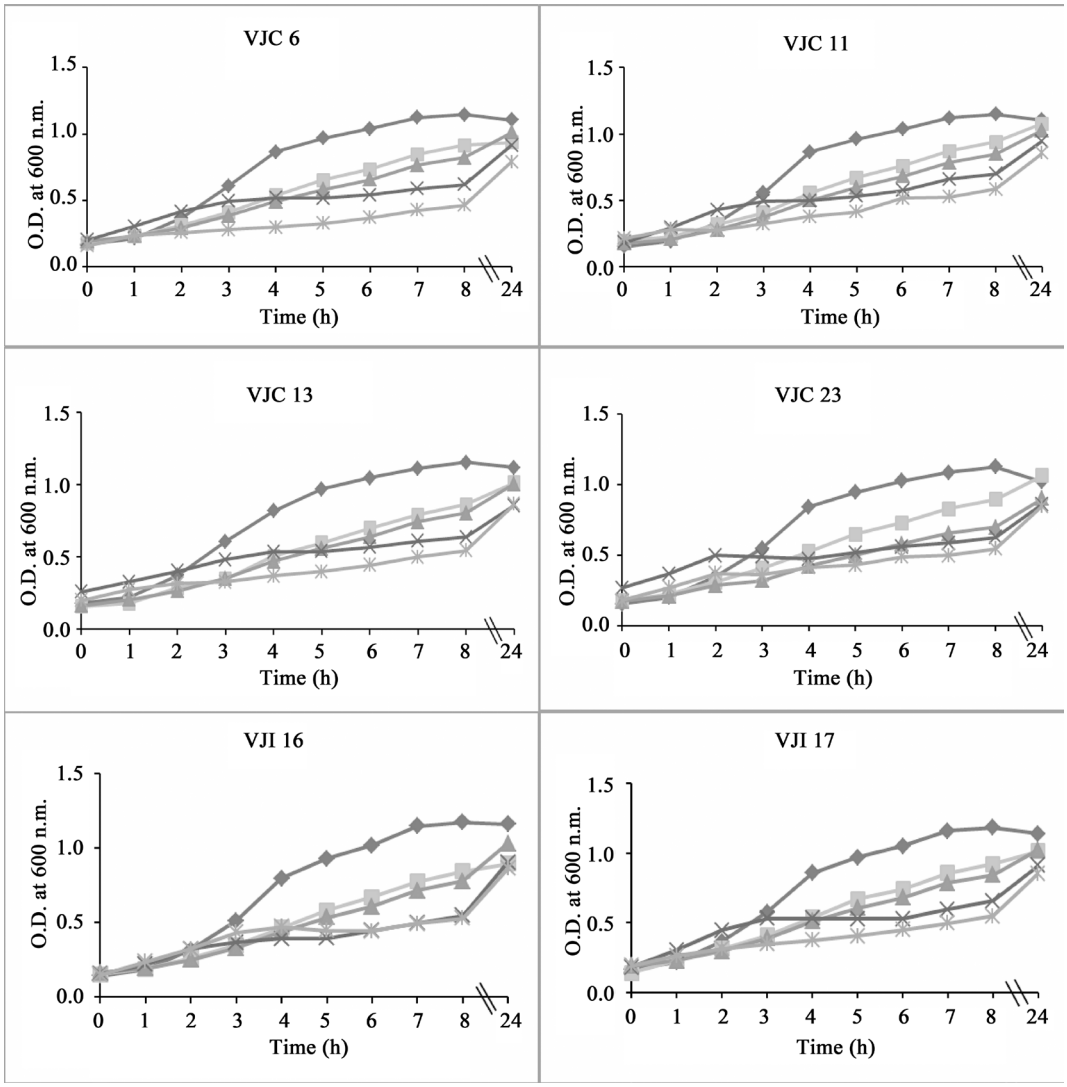


Figure 6. Comparison of growth of VJC6 (A), VJC11 (B), VJC13 (C), VJC23 (D), VJI16 (E) and VJI17 (F) in MRSbroth at different bile concentrations.

Table 3. Probiotic properties of isolates.

Properties	VJC6	VJC11	VJC13	VJC23	VJI16	VJI17
Autoaggregation (%)	86.21 ± 0.50	86.28 ± 0.48	85.36 ± 1.08	86.50 ± 0.64	86.92 ± 0.88	78.97 ± 0.86
Coaggregation with *L.monocytogenes* (%)	38.78 ± 0.40	30.00 ± 0.70	41.27 ± 0.50	36.02 ± 0.40	34.50 ± 0.27	45.83 ± 0.50
Coaggregation with *Escherichia coli* (%)	56.57 ± 0.40	55.52 ± 0.35	61.49 ± 1.50	49.38 ± 0.50	49.38 ± 0.50	51.77 ± 1.17
Cell-surface hydrophobicity (%)	29.80 ± 0.23	33.29 ± 0.84	16.35 ± 0.32	45.16 ± 0.25	29.41 ± 0.12	28.70 ± 0.24
β-galactosidase activity (MU)	1074 ± 7	1059 ± 10	1179 ± 5	1166 ± 11	1036 ± 5	1106 ± 6
Bile salt hydrolase activity	+	+	+	+	+	+
Hemolytic activity	−	−	−	−	−	−

Mean (± standard deviation) of results of three experiments, +: positive; −: negative.

concentrations of bile (**Figure 6**). Bacterial cell hydrophobicity may assist in adhesion, but does not seem to be a prerequisite for colonization [34]. The *L. plantarum* strains showed hydrophobicity ranging from 16% - 33% with n-hexadecane. Lactose intolerance is caused by deficiency β-galactosidase in the lining of duodenum. So, the food products, fermented with lactase producers could help to treat lactose intolerance. All the isolates showed β-galactosidase activity. Among the isolates, VJC13 showed maximum β-galactosidase activity (1179 MU) may have application in the dairy industry. In this study no isolate showed hemolytic activity where as positive strains *Escherichia coli* MTCC 728 and *Streptococcus pyogenes* MTCC 442 showed α- and β-hemolysis, respectively. **Table 3** gives the probiotic properties of the all the *Lactobacillus* strains.

4. Conclusion

In conclusion, chicken GIT is a good reservoir of potential antifungal, antibacterial and probiotic *Lactobacillus plantarum* strains with high diversity. The study of antifungal nature and probiotic properties *in vivo* of these isolates may lead to the development of lactic acid bacteria based preservative systems to prevent fungal spoilage in food and feed industries.

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Supplementary Figures

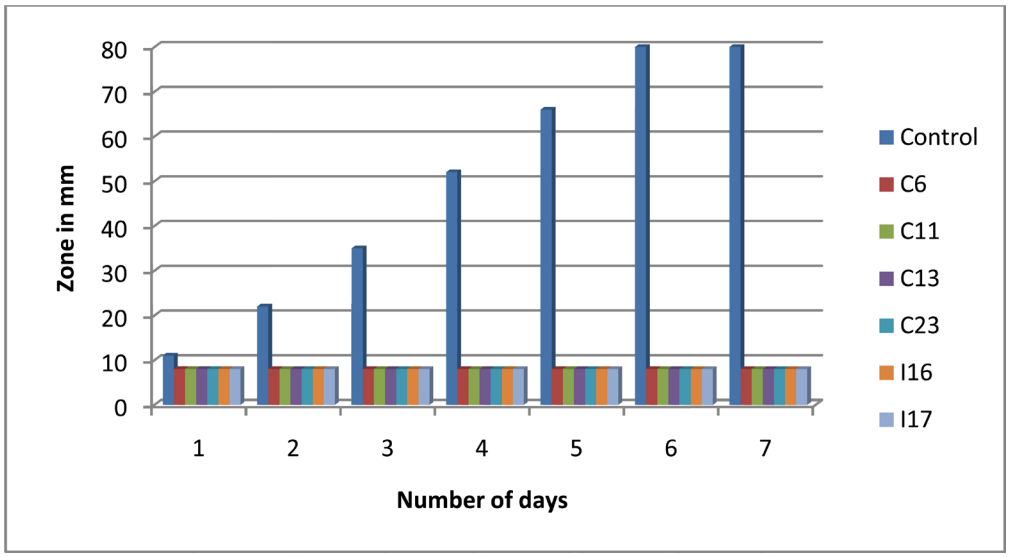

Figure S1. Inhibition of *Aspergillus niger* MTCC 4325 by *Lactobacillus* isolates in a dual agar layer system.

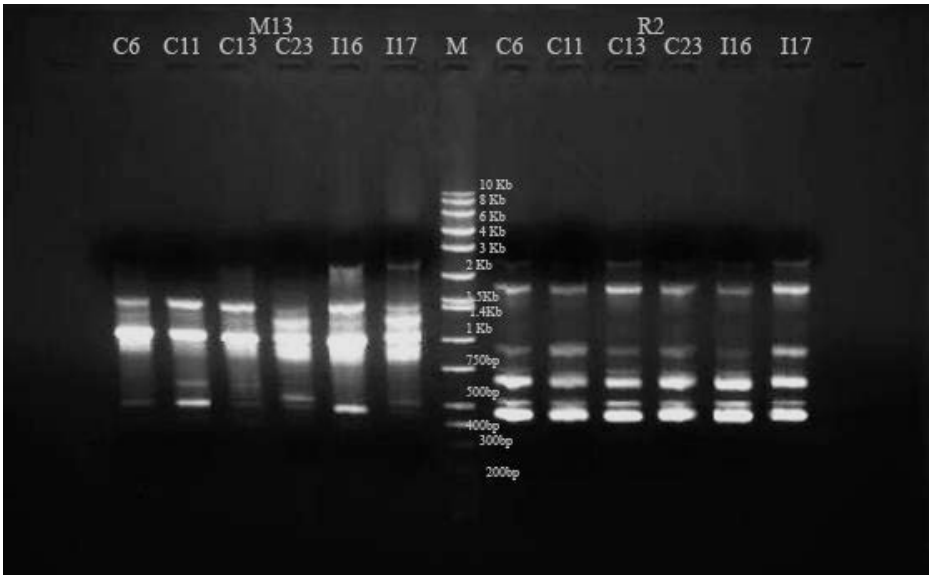

Figure S2. M13 and R2 RAPD analysis of six potent strains.

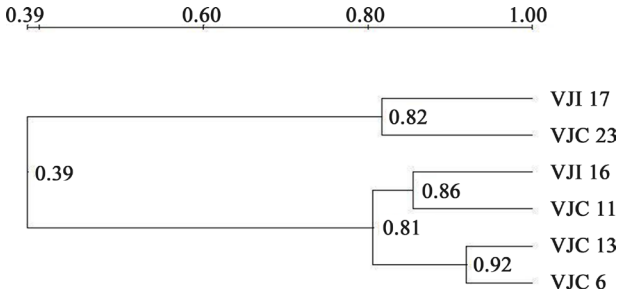

Figure S3. Dendrogram based on the UPGMA clustering analysis and the Dice coefficient of the M13 RAPD patterns.

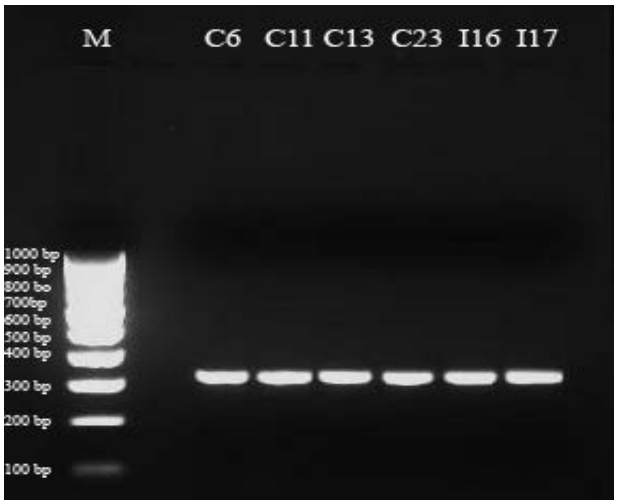

Figure S4. Multiplex PCR of six isolates.

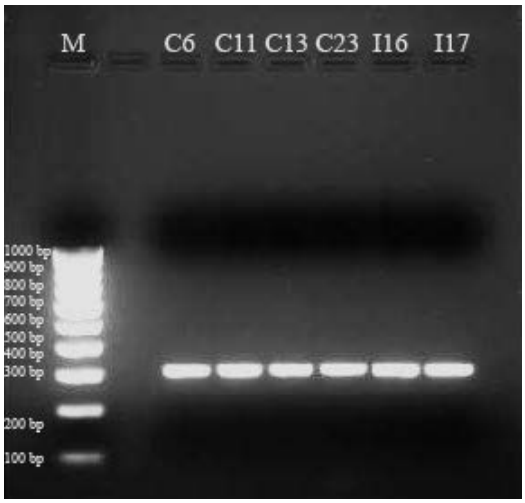

Figure S5. PCR amplification of plnEF gene of six potent strains.

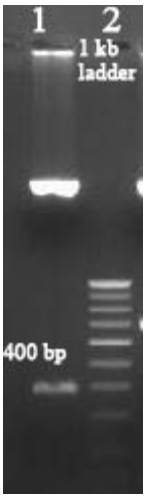

Figure S6. *EcoR* I and *Hind* III digestion of recombinant vector pTZ57R/T with plnEF gene. 1) *EcoR* I and *Hind* III double digest product, 2) 1 kb DNA ladder.

Supplementary Tables

Table S1. Biochemical characterization of the isolates.

	VJC6	VJC11	VJC13	VJC23	VJI16	VJI17
Carbohydrate Utilization						
Lactose	+	+	+	+	+	+
Xylose	–	–	–	–	–	–
Maltose	–	+	+	+	+	+
Fructose	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+
Galactose	±	±	±	±	±	±
Raffinose	–	–	–	–	–	–
Trehalose	+	+	+	+	+	+
Melibiose	–	–	–	–	–	–
L-Arabinose	–	–	±	+	–	±
D-Arabinose	±	–	–	±	–	–
Mannose	+	+	+	+	+	+
Inulin	+	+	+	+	+	±
Sucrose	+	+	+	+	+	+
Glycerol	–	–	–	–	–	–
Sorbitol	±	–	–	–	–	–
Mannitol	±	–	–	–	–	–
Salicin	±	–	+	+	+	–
Esculin hydrolysis	+	+	+	+	+	+
Citrate utilization	–	–	–	–	–	–
Sodium gluconate	–	–	–	–	–	–
Cellobiose	+	+	+	+	+	±
Erythritol	–	–	–	–	–	–
Melezitose	+	+	+	+	+	±
α-methyl-D-mannoside	–	–	–	–	–	–
α-methyl-D-glucoside	–	–	–	–	–	–

Table S2. Antibiotic susceptibility profile of isolates from chicken GIT.

Antibiotics	C6	C11	C13	C23	I16	I17
Cell wall synthesis inhibitor						
Penams						
Ampicillin (25 µg)	S	S	S	S	S	S
Cloxacillin (5 µg)	S	S	S	S	S	S
Penicillin G (2 µg)	S	S	S	S	S	S
Cephams						
Cephalexin (30 µg)	R	R	R	S	R	S
Cephaloridine (30 µg)	R	R	R	R	R	R
Cepharadine (30 µg)	R	R	R	R	R	R
Cefuroxime (30 µg)	R	S	R	S	S	S
Ceftriaxone (30 µg)	R	S	R	R	R	S

Continued

Others						
Mecillinam (33 µg)	S	S	S	S	S	S
Nucleic acid synthesis inhibitor						
Norfloxacin (300 µg)	R	R	R	R	R	R
Ciprofloxacin (10 µg)	R	R	R	R	R	R
Nalidixic acid (30 µg)	R	R	R	R	R	R
Co-trimoxazole (25 µg)	S	S	S	S	S	S
Protein synthesis inhibitor						
Amikacin (10 µg)	R	R	R	R	R	R
Erythromycin (10 µg)	S	S	S	S	S	S
Gentamycin (30 µg)	R	S	S	R	S	S
Lincomycin (10 µg)	S	S	S	S	S	S
Streptomycin (10 µg)	R	R	R	R	R	R
Tetracyclin (25 µg)	S	S	S	S	S	S
Chloramphenicol (25 µg)	S	S	S	S	S	S
Urinary tract antiseptics						
Nitrofurantoin (300 µg)	R	R	R	R	R	R
Cytoplasmic membrane function inhibitors						
Colistin (10 µg)	R	R	R	R	R	R