CHAPTER 4

INHIBITION OF BIOFILM FORMATION OF *LISTERIA MONOCYTOGENES* BY BACTERIOCIN - PRODUCING *PEDIOCOCCUS PENTOSACEUS* DS1 ON SELECTED BIOTIC AND ABIOTIC SURFACES

Inhibition of *Listeria monocytogenes* biofilm formation by bacteriocin - producing *Pediococcus pentosaceus* DS1 on selected biotic and abiotic surfaces

4.1. Abstract

The present study explores the antilisterial activities of potentially probiotic strain *Pediococcus pentosaceus* DS1 isolated from fermented bamboo shoot. 16s rRNA gene sequence analysis confirmed the identity of the strains. Class IIA bacteriocin pediocin expressing YGNGV motif was identified and its antilisterial activity was validated by well diffusion assay. When cocultured with *P. pentosaceus* DS1, in case of *L. monocytogenes* AMDK2 maximum biofilm exclusion (55.54 ± 3.44%) was observed, which could be visually observed by scanning electron micrographs. *P. pentosaceus* DS1 showed maximum adhesion inhibition to Caco-2 cell line in case of *L. monocytogenes* AMDK2 (91.8%), whereas maximum decrease in invasion was observed in case of *L. monocytogenes* MTCC 839 (52.9%). Coculture in UHT milk also significantly decreased microbial growth parameters of *Listeria* suggesting the efficiency of *P. pentosaceus* DS1 in the removal of *Listeria* contamination from food.

4.2. Introduction

Pathogenic bacteria have the capability to withstand the deleterious conditions prevailing in the host body through the mechanism of biofilm formation. Biofilms are described as cells held together by extracellular polymeric substances adhered to a nutrient- rich surface forming a multi- layered microbial community [1]. The initial attachment of bacterial cells is followed by the production of polymeric substances and once established, biofilms have the capability to release planktonic cells to start colonizing in new substratum [2].The prevalence of microbial biofilm is very diverse and is a major concern in food, biotechnological, marine, medical and other industrial fields. Biofilm is the most common mode of bacterial growth in the environment and has the capability to resist antibiotics, biocides, UV radiation more efficiently than the planktonic cells.

Microbial contaminations in food industries lead to the spoilage of foodstuffs accompanied by food poisoning and ultimately results in economic loss and food-borne diseases. According to centres of disease control and prevention (CDC) report,

every year nearly 48 million people suffer from food-borne illness, 128,000 are hospitalized, and 3,000 die in United States [3]. *Listeria monocytogenes* is an opportunistic food- borne pathogen which is responsible for the third highest deaths caused by food-borne bacteria [4]. It is capable of formation of biofilms on various food contact surfaces [5] and can persist for a long period. Similarly, it has also the capability to adhere to the gastrointestinal cells of the host, thereby increases the probability of infections.

Therefore demand for food safety is the foremost concern for every individual and country. Due to consumer's concern about the possible ill effects of chemical preservatives present in processed foods, search for novel preservation techniques involving minimal processing with enhanced shelf-life is nowadays a trend. The application of natural antimicrobial peptides has claimed much attention towards prevention of food spoilage that inhibits food pathogens without undesirable effects. Recently, the focus is on class II bacteriocins since they show activity against food pathogens including *Listeria monocytogenes, Staphylococcus aureus* etc [6]. Pediocin is a class IIa bacteriocin containing the 'YGNGV' motif and two extra C- terminal disulphide bonds which make pediocin more potent bacteriocin [7].

Traditional fermented foods have been reported to harbour lactic acid bacteria (LAB) producing natural antimicrobial peptides [8] that exhibit antagonistic activity against various food-borne pathogens. North-East India is home to various cultural groups and tribes, having profound knowledge of fermented food preparation including *Khorisa* (fermented bamboo shoots) consumed by the ethnic people of Assam, India. Nearly 19 genera with more than 78 species of native and exotic varieties of bamboo are cultivated in the biodiversity regions of Northeast India [9]. The present study focuses on the characterization of a newly isolated bacteriocin producing LAB strain *Pediococcus pentosaceus* DS1 from *Khorisa* and its ability to inhibit pathogen adhesion to the gut as well as biofilm formation. Moreover, the inhibition of *Listeria* in UHT milk also gives insight into an alternative strategy of pathogen inhibition.

4.3. Materials and methods

4.3.1. Bacterial strains, media and growth conditions

The strain DS1 was isolated from fermented bamboo shoot, *Khorisa,* presumptively identified as *Pediococcus pentosaceus* utilizing different biochemical tests as described in chapter 3. *Listeria monocytogenes* AMDK2 was isolated from spoiled foodstuffs in Industrial and Applied Microbiology Laboratory, Tezpur University. *L. monocytogenes* MTCC 839 and *Lactobacillus plantarum* MTCC 1407 which was used as a standard probiotic strain were purchased from Microbial Type Culture Collection (MTCC), IMTECH, India. *P. pentosaceus* DS1 and *L. plantarum* MTCC 1407 was grown in de Man, Rogosa and Sharpe (MRS) media and *L. monocytogenes* strains were grown in tryptic soy broth (TSB) at 37 °C.

4.3.2. Identification of bacterial strains

The isolates were identified by 16S rRNA gene sequencing followed by phylogenetic tree construction. Universal primers analysis followed by phylogenetic studies. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) were used for the amplification of 16S rRNA gene sequence [10]. The conditions for PCR amplifications were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of primer annealing for 30 s at 53 °C, elongation for 1 min at 72 °C, and final 10 min extension at 72 °C [11]. PCR product was separated by electrophoresis in 1% (w/v) agarose gel. The amplified PCR product was purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence was analyzed using BLAST algorithm (http:// www.ncbi.nlm.nih.gov/blast) and was submitted to the NCBI GenBank (http:// www.ncbi.nlm.nih.gov/genbank). The phylogenetic tree was constructed by neighbor- joining (NJ) method using MEGA 5.05 software [12, 13, 14].

4.3.3. Assessment of probiotic properties of the isolates

4.3.3.1. *In vitro* gastrointestinal stress tolerance

The tolerance of the isolates to simulated gastric and intestinal conditions was evaluated using a protocol reported by Maragkoudakis et al. 2006 [15] with some modifications. Simulated gastric juice (SGF) was prepared by adding filter- sterilized 0.3 mg/ml pepsin to PBS with pH adjusted to 2, 3 and 4. Simulated intestinal fluid (SIF) was prepared by adding 0.1mg/ml pancreatin and 0.3% (w/v) bile salt to PBS adjusted to pH 6.8 and 8. Isolates were cultured in their respective media and overnight cultures were harvested by centrifugation at 6000 X g and the cells were washed with phosphate buffered saline (PBS, pH 7.4). Cell suspension in PBS (pH 7.4) was added to PBS solutions with different pH values prepared beforehand at a final concentration of ~10^8 CFU/ml. For SGF, cells were incubated for 1, 2 and 3 h and for SIF incubation was done for 1, 2, 3 and 4 h. Viability was checked after 24 h incubation at 37° C by plate count method.

Viability was checked according to the formula given below:

$$\text{Viablility}(\%) = \frac{\log \text{CFU/ml}_{\text{final}}}{\log \text{CFU/ml}_{\text{initial}}} \times 100 \quad (1)$$

Where, $\text{CFU/ml}_{\text{initial}}$ is the viability at 0 h and $\text{CFU/ml}_{\text{final}}$ is the viability at the end of the experiment.

4.3.3.2. Adhesion to Caco-2 cell line

The human colorectal adenocarcinoma Caco-2 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. The cell line was routinely grown and maintained in minimal essential medium (MEM) supplemented with 20% heat- inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, and grown at 37 ºC in humified atmosphere in presence of 5% (v/v) CO_2. Cells were supplemented with fresh MEM every 2-3 days.

Adhesion assay was performed using a method described by García-Cayuela et al. 2014 [16]. Briefly, Caco-2 cells were seeded onto 24 well tissue culture plates at a concentration of 10^4 cells per ml and incubated in humified atmosphere containing 5% CO_2 at 37° C. Media was changed in every two days until cells become 80%

confluent. After cells attain confluence, they were washed with phosphate buffered saline (PBS, pH 7.4) and replenished with new media containing no antibiotics. Now the monolayer cells were inoculated with probiotics (10:1 ratio of probiotics to Caco-2 cells) and incubated for 1 h. After the incubation period was over, media was discarded and the wells were washed with PBS to remove the non-adhered cells. Some wells were stained with crystal violet and observed under microscope (100X). The other wells were trypsinized with 0.25% trypsin-EDTA solution and viable bacterial count was determined using plate count on MRS agar.

Adhesion was calculated by the following formula

$$\text{Adhesion}(\%) = \frac{\log \text{CFU/ml}_{\text{final}}}{\log \text{CFU/ml}_{\text{initial}}} \times 100 \quad (2)$$

4.3.4. Detection of bacteriocin production gene

Gene encoding pediocin was amplified using the primers and PCR conditions reported by Sood S. K. et al. [17] with some modifications. Briefly, PCR was performed in 25 µl volume containing 12.5 µl PCR Master Mix (Thermo Fisher Scientific), 9.5 µl MilliQ water, 1 µl each of forward primer 5´-TGGCCAATATCATTGGTGGT-3´ and reverse primer 5´-CTACTAACGCTTGGCTGGCA-3´. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 32 cycles of final denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 45 s and final extension at 72 °C for 4 min and cooling to 4 °C.

The detection of antimicrobial activity of bacteriocin was performed by following the method of Cocolin et al. (2007) with slight modifications [18]. The cell free supernatant (CFS) obtained after centrifugation of overnight culture was filtered and neutralized (pH 7.0) to eliminate the effects of acids. CFS was also treated with proteinase K and catalase to see whether the activities are due to proteins or free radicals. Clear zones of inhibition around the wells were measured which indicated the antimicrobial properties.

4.3.5. Autoaggregation assay

Autoaggregation assay of the probiotic and pathogenic strains were performed according to the method reported by Del Re et al. [19]. Briefly, 4 ml of cell suspension (10^9 CFU/ml) was vortexed for 10 sec and incubated at 37 ºC. After 4 hours, a 100 µL aliquot was taken out from the upper surface, mixed with 900 µL of PBS (pH 7.4) and OD_{600} was measured. Autoaggregation percentage was calculated as:

$$\text{Autoaggregation } (\%) = \left(1 - {}^{A_t}/_{A_0}\right) \times 100 \quad (3)$$

4.3.6. Bacterial adhesion to solvent assay

The affinities of isolates towards solvents were assessed following the method of Rosenberg, 2006 [20]. Cells from a previously grown culture were harvested and washed twice with PBS, pH 7.4. Cell count was adjusted approximately to 10^9 CFU/ml. 2 ml of cell suspension was mixed with equal volume of xylene, chloroform or ethylene acetate by vortexing for 2 minutes. The aqueous and the organic phases were allowed to separate by keeping the mixture undisturbed for 1 hour. After that, the aqueous layer was gently pipetted out and OD_{600} was measured. The bacterial adhesion to solvents was calculated as:

$$\text{Hydrophobicity}(\%) = \left(\frac{Abs_{initial} - Abs_{final}}{Abs_{initial}}\right) \times 100 \quad (4)$$

Where $Abs_{initial}$ represents initial absorbance before mixing and Abs_{final} represents final absorbance after mixing with solvents.

4.3.7. Probiotic mediated biofilm inhibition assay

Biofilm inhibition assay was performed according to a protocol reported by Woo et al. [21] with some modifications. All bacterial cultures mentioned here were maintained at 10^7 CFU/ml. Three different assays were performed, i.e. competition, exclusion and displacement. Initially, for the competition assay, both probiotic and pathogenic strains were cocultured in 12 well microtiter plates in TSB and incubated for 24 h at 37 ºC. For the exclusion assay, pathogens were added to pre-grown probiotic culture in 12 well plates and incubated for 24 h at 37 ºC. For the displacement assay, probiotic cultures were added to pre- formed biofilm of pathogens and incubated for 24 h at 37 ºC. Monoculture of pathogens was also incubated under the same

conditions as controls. After the incubation period, wells were washed with phosphate buffered saline (PBS), pH 7.4 to remove the non- adherent cells. Biofilm cells were removed using a cell scrapper and serially diluted. Properly diluted cells were spread on Oxford Listeria Agar, incubated at 37 ºC for 24-48 h and plate count was carried out. Biofilm inhibition was calculated by the following formula

$$\text{Biofilm inhibition (\%)} = \frac{\log \text{CFU/ml}_{\text{treated}}}{\log \text{CFU/ml}_{\text{control}}} \times 100 \quad (5)$$

Where, CFU/ml $_{\text{control}}$ is the cell count of monoculture and CFU/ml treated is the cell count of probiotic- treated wells.

4.8.8. Scanning electron microscopic observations of biofilm inhibition

Biofilm inhibition was observed according to a method described by Melo et al. [22] with some modifications. The culture supernatant of *P. pentosaceus* DS1 was filter sterilized with 0.22 µm syringe filter and then lyophilized. Then minimum inhibitory concentration (MIC) of the lyophilized product was determined against both the strains of *L. monocytogenes* by well diffusion method. After that, lyophilized supernatant was added to TSB media in the wells of a 12 well tissue culture plate at a concentration of 50% MIC value and inoculated with the *L. monocytogenes* strains. Wells without lyophilized supernatant were also taken as control wells. To each well, a glass coverslip was dipped. After incubation for 24h at 37 ºC, coverslips were rinsed with PBS to remove unbound cells and then fixed with 2.5% glutaraldehyde for 6 h and washed twice with 1X PBS, pH 7.4. Further, samples were dehydrated in graded concentrations of ethanol. Then specimens were platinum coated using JEOL JFC-1600 auto fine coater and observed under SEM (JEOL model JSM-6390 LV) at 15 kV.

4.3.9. Effect of probiotics on Caco-2 cell adhesion of *Listeria monocytogenes*

For the determination of adhesion inhibition of *Listeria monocytogenes* to Caco-2 cell line, cells were first grown in 24 well tissue culture plates till confluence. The spent media was discarded and monolayers were washed with PBS. Probiotic and pathogen mixture at a concentration of 10^8 CFU/ml each were inoculated into the wells containing antibiotic-free MEM and incubated for 2h [23]. Control wells were also kept inoculated with pathogen only at a concentration of 10^8 CFU/ml. After incubation is over, the spent broth was discarded and monolayer cells were treated

with 0.05% trypsin- EDTA. Cells were serially diluted and plated on Oxford *Listeria* Agar for viable count.

4.3.10. Effect of probiotics on Caco-2 cell invasion of *Listeria monocytogenes*

Caco-2 cell invasion assay was performed according to a protocol reported by Handa-Miya et al. [24] with some modifications. Confluent Caco-2 cells containing antibiotic- free MEM were inoculated with 10^9 CFU/ml of probiotic and incubated for 1 h. After 1h, 10^7 CFU/ml of pathogens were added to the wells and incubated further for 2h at 37 ºC. After incubation, non- adherent bacterial cells were washed away with PBS. The adherent bacterial cells were killed by incubating for 1 h in media containing 50 mg/ml gentamicin (SigmaeAldrich). The Caco-2 cells were then lysed with 1% triton X-100 and viable count of the invading *L. monocytogenes* were calculated in Oxford *Listeria* Agar.

4.3.11. Antagonistic effect of *P. pentosaceus* DS1 *L. monocytogenes* in milk

Commercial ultra high temperature processed (UHT) milk was purchased from local market. Growth curves were generated according to a protocol described by Aguilar et al. [25]. Briefly, 5 ml of UHT milk in screw cap tubes were inoculated with either *L. monocytogenes* culture alone or *P. pentosaceus* DS1and *L. monocytogenes* in a ratio of 1:1. The initial count of each bacterium was set at 2.5 log CFU/ml. All experiments were carried out in triplicates at 37 ºC in non- agitated conditions. Viable counts of *L. monocytogenes* in monocultures or in cocultures were carried out by spreading on *Listeria* Oxford Agar. pH of the culture media was also monitored routinely. Growth parameters such as initial and maximum population level, lag time, and maximum specific growth rate were calculated by fitting the growth data into The Baranyi & Roberts model [26] using the DMfit curve-fitting program (available at www.ifr.bbsrc.ac.uk/safety/DMFit), version 3.5.

4.4 Results and discussions

4.4.1 Strain identification

The 16S rRNA gene sequencing and phylogenetic analysis of DS1 revealed close relationship to *Pediococcus pentosaceus* strain E24-168 (Fig. 4.1.) and sequences were submitted to NCBI GenBank as *Pediococcus pentosaceus* DS1 (KP723364).

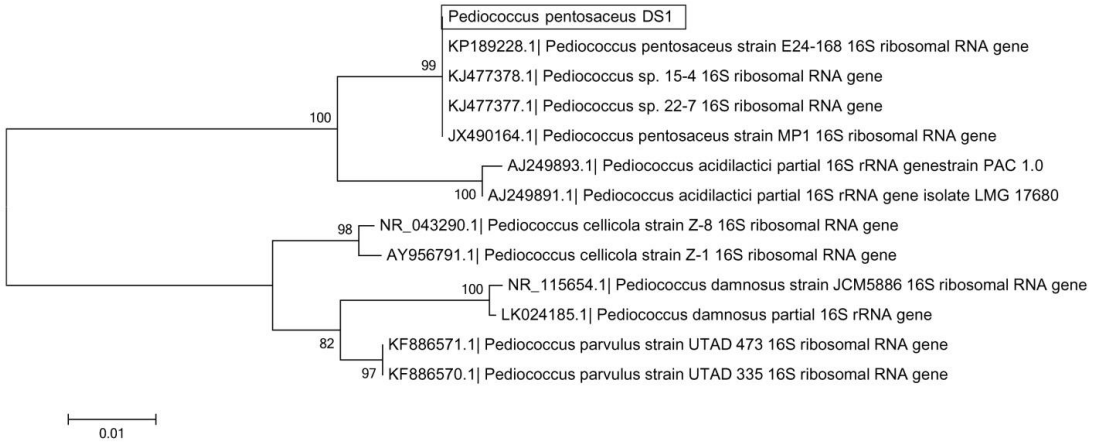


Fig. 4.1. Phylogenetic tree showing DS1 with closely related species based upon 16S rRNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes.

After sequencing, the isolate AMDK2 showed 99% similarity to *Listeria monocytogenes* and the phylogenetic tree is as shown in the Fig. 4.2.

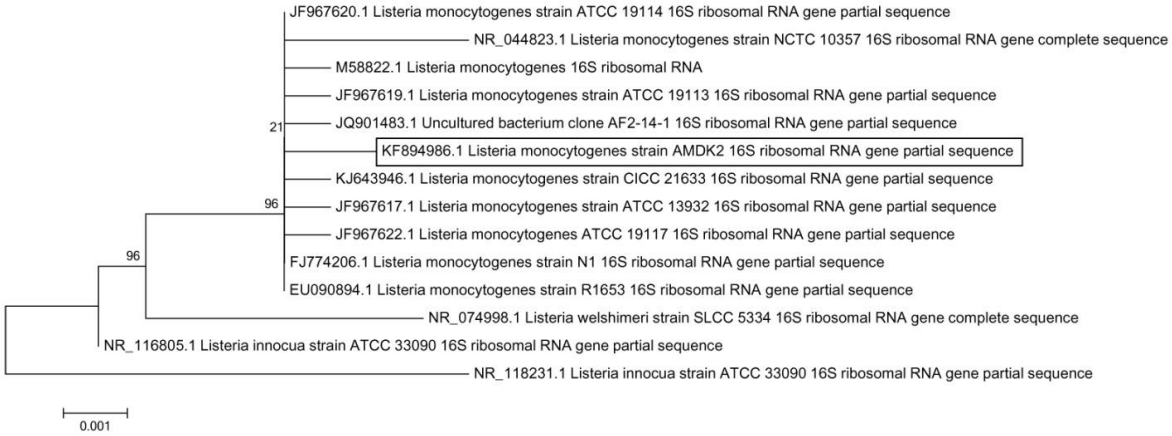


Fig 4.2. Phylogenetic tree showing AMDK2 with closely related species based upon 16S rRNA gene sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes.

4.4.2. Probiotic characterization

4.4.2.1 Tolerance to simulated gastrointestinal transit

Tolerance to gastrointestinal conditions is a much needed trait for probiotics since they have to pass through the gastrointestinal barrier along with food matrix to deliver their health beneficial activities. In the present study the viability of the probiotics was evaluated in conditions which simulate the physiological environment prevailing in the gastrointestinal tract. This was accomplished by varying the pH values of the incubation buffer and the addition of proteolytic enzymes such as pepsin and

pancreatin. The incubation time for SGF and SIF were 3 and 4 h respectively which reflected the time spent in the stomach and small intestine [15]. As shown in the table 4.1, the both the strains retained viability more than 80% in case of both SGF and SIF. In pH 2 and pH 3, a slight decrease in viability was observed, where in both strains viability was in the range of 80-90%. Otherwise, viability was found to be always more than 90%. These results are in agreement with previous reports [27, 28].

Table 4.1. Tolerance to simulated gastrointestinal transit tolerance; (A) simulated gastric fluid tolerance, (B) simulated intestinal fluid tolerance

(A) SGF tolerance (viability in %)						
Time (h)	pH 2		pH 3		pH 4	
	DS1	MTCC 1407	DS1	MTCC 1407	DS1	MTCC 1407
1	87.84±0.902	88.46±0.497	89.59±0.942[a]	88.52±0.113	102±3.103	95.99±1.202
2	85.76±0.291	84.4±0.402	86.08±1.044[b c]	84.73±0.475	101.5±2.394	99.5±1.69
3	86.42±0.395	84.61±4.948	88.48±0.330[a c]	84.36±1.84	103.92±0.579	98.44±1.29
(B) SIF tolerance (viability in %)						
Time (h)	pH 6.8		pH 8			
	DS1	MTCC 1407	DS1	MTCC 1407		
1	101±1.778	95.96±2.242	98.64±1.492	96.38±1.041		
2	100.67±0.707	99.57±2.084	102±1.145	90.53±0.19		
3	100.84±1.567	96.43±1.71	101.55±0.169	93.36±3.22		
4	98.41±0.458	98.23±0.976	98.44±0.043	96.88±0.962		

Values are represented as mean ± SD., n=6. Means with different letters in the same row are significantly different (P<0.05), checked by Tukey's multiple comparison test, GraphPad prism, ver. 5.0

Abbreviations: DS1- *Pediococcus pentosaceus* DS1, MTCC 1407- *Lactobacillus plantarum* MTCC 1407

4.4.2.2 Adhesion to Caco-2 cell line:

The ability to adhere to human intestinal cells is another essential criterion for probiotic selection which reflects successful colonization for the exertion of health modulating effects. Caco-2 cell line is the most extensively used model system for the assessment of probiotic adhesion. The adhesion of probiotics can be visualized from gram staining of probiotic- treated Caco-2 and the untreated Caco-2 cell (Fig. 4.3). From the quantitative assay of cell adhesion it was found that maximum adhesion was shown by the strain *Pediococcus pentosaceus* DS1 (18.15%) as shown in the Fig.

4.3D which was not significantly different from the adhesion of the probiotic reference strain *Lactobacillus plantarum* MTCC 1407. Probiotic adhesion is a strain specific process and *Pediococcus* strains are reported to show variable adhesion towards Caco-2 cell (29, 30, 31)

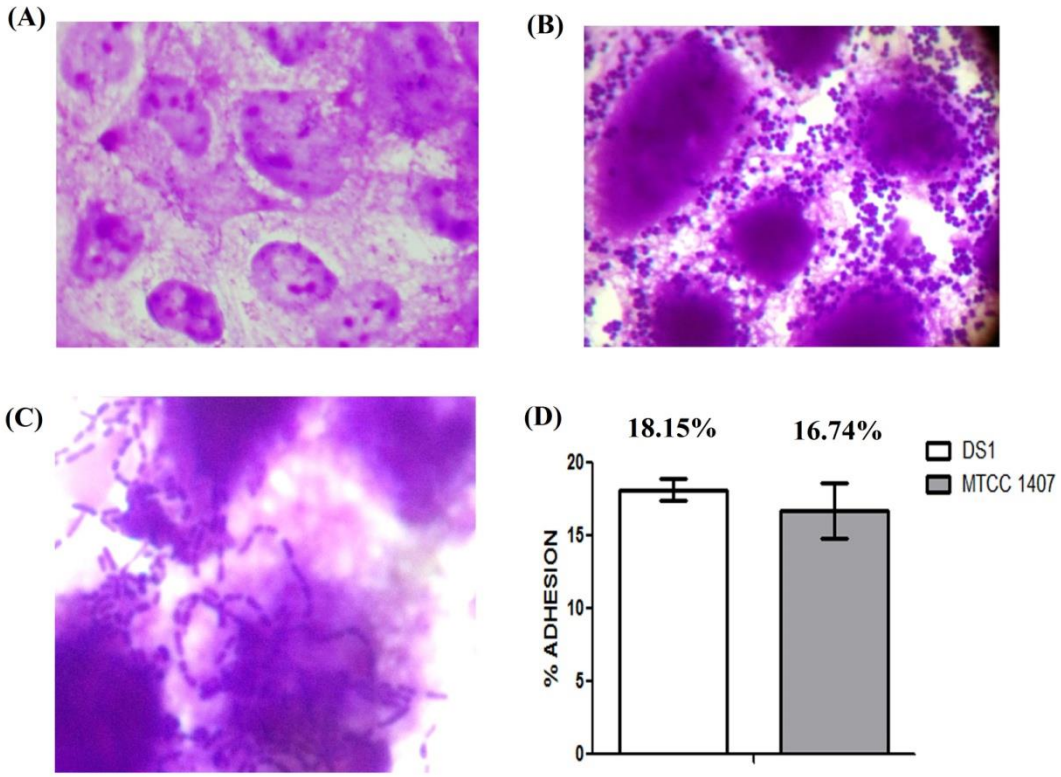


Fig. 4.3. Probiotic adhesion to Caco-2 cell line; (A) control Caco-2 cell, Gram staining depicting adhesion of (B) *P. pentosaceus* DS1 and (C) *L. monocytogenes* MTCC 1407 observed under 100X, (D) Quantitative representation of adhesion showing percentage adhesion expressed as bar diagram.

4.4.3. Detection of bacteriocin producing gene

Pediococcus pentosaceus isolated from different fermented food products are known to produce the bacteriocin pediocin [32]. In our study, the primers which were designed based on the novel method of Sood et al. [17] could amplify the YGNGV motif-containing pediocin gene. The size of the PCR product was found to have a size of 406 bp (Fig. 4.4A). The deduced amino acid sequence showed 100% similarity with pediocin PA-1 (Fig. 4.4B). The multiple sequence alignment also reveals the conserved motif YGNGV (Fig. 4.5A). The nucleotide sequence was submitted to GenBank and received an accession number KT345707.

Among different servers that predict the secondary structure of protein, PSIPRED is the most accurate one that employs two forward neural networks to analyze the outputs obtained from PSI- BLAST [33]. The secondary structure analysis reveals the presence of multiple strand- loop structures (Fig. 4.5B). Starting from the YGNGV bacteriocin IIa motif, three short β strands connected by short loop or turn followed by a helix at the C terminus was observed. Previous works on the secondary structure of class IIa bacteriocins yielded similar results [34].

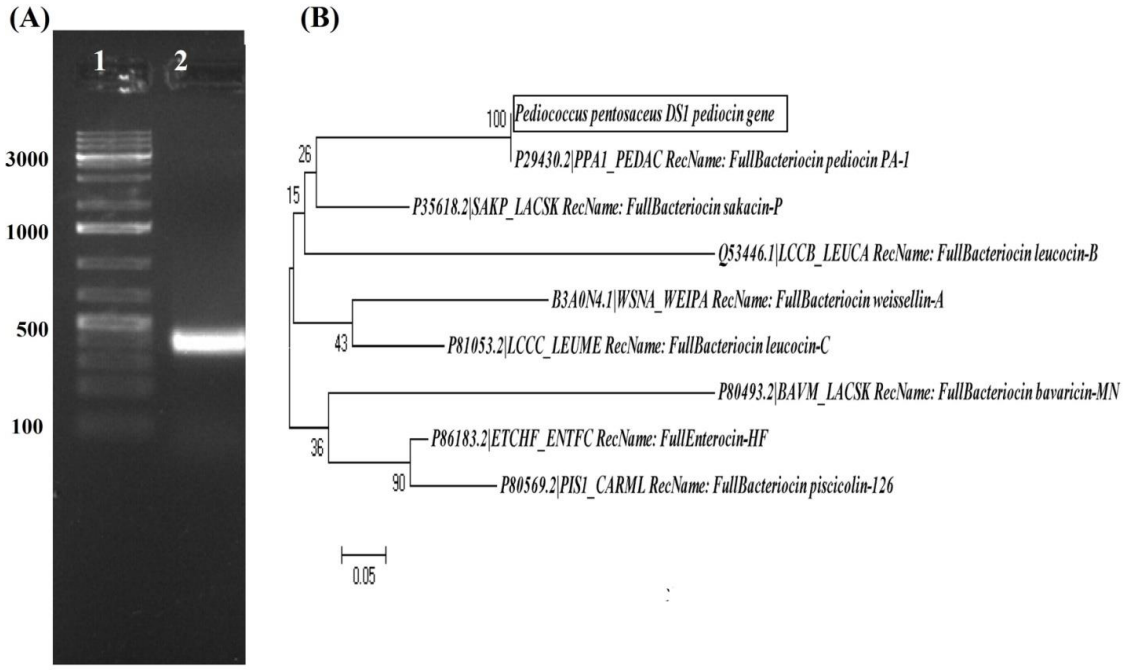


Fig. 4.4. (A) Amplification of pediocin gene; lane 1: 1Kb+ ladder, lane 2: Amplicon of size 406 bp. (b) Phylogenetic tree showing *P. pentosaceus* DS1pediocin with closely related sequences.

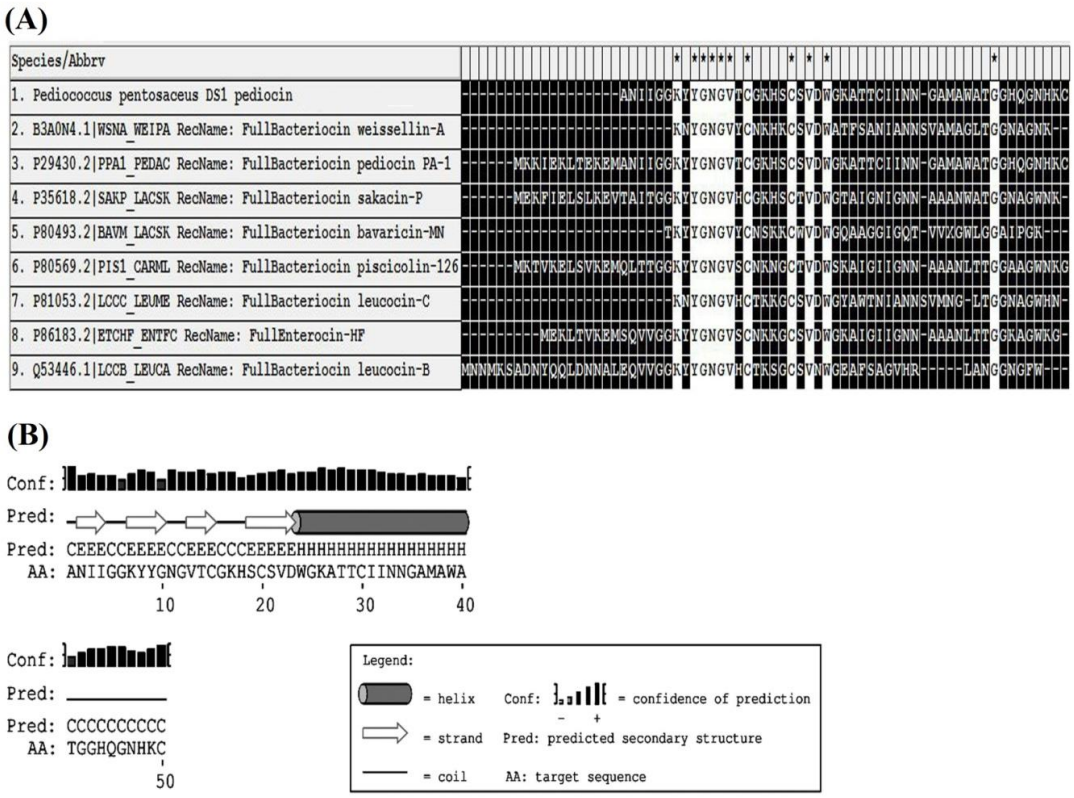
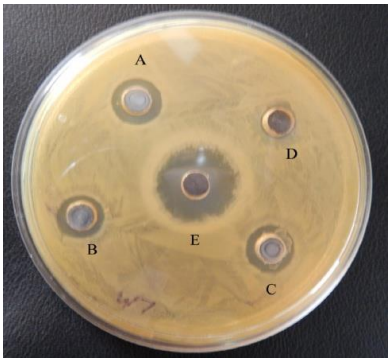


Fig. 4.5. (A) Multiple sequence alignment of *Pediococcus pentosaceus* DS1 pediocin gene (B) Secondary structure deduced from PSIPRED server.

4.4.4. Detection of bacteriocin production in culture media

As depicted in the Fig. 4.6 it is clear that when the CFS was neutralized to pH 7.0 or treated with catalase, antibacterial activity persisted whereas treatment with proteinase K resulted in the loss of activity. Therefore the components responsible for the inhibition of food spoilage indicator strains must be due to some proteinaceous bacteriocinogenic activity.



4.6. Antimicrobial activity of culture supernatant against *Listeria monocytogens* MTCC 839; (A) control (untreated), (B) pH neutralized, (C) catalase treated, (D) proteinase K treated and (E) antibiotic (ampicillin)

4.4.5. Autoaggregation and bacterial adhesion to solvent assay

The autoaggregation properties of probiotic *P. pentosaceus* DS1 and the pathogens, i.e. *L. monocytogenes* MTCC 839 and AMDK2 are given in the Fig. 4.7A. Both autoaggregation properties and bacterial adhesion to solvents are correlated with the adhesion properties of bacteria to the epithelial cells. In our experiments it was found that the highest autoaggregation was shown by the strain DS1 (50.85 ± 1.29%) followed by *L. monocytogenes* MTCC 839 (48.33 ± 4.38%). The autoaggregation abilities of the probiotic strain was not found to be statistically different from the pathogenic strains ($P<0.5$). From the bacterial adhesion to solvent assay (Fig. 4.7B) it was found that in all strains, maximum adhesion was found towards chloroform indicating acidic and electron donor nature of the affinity, while poor electron acceptor nature was revealed by low affinity towards ethyl acetate. These results support the findings of Woo et al. [19]. Cell surface hydrophobicity is a very important trait which plays an important role cell adhesion and biofilm formation [35].The affinities towards apolar xylene indicates hydrophobicity of the bacterial cell wall and maximum hydrophobicity was shown by the strain DS1 (66.47 ± 4.95%) followed by *L. monocytogenes* MTCC 839 (48.33 ± 4.38%). These values were statistically not significantly different; indicating that pathogen *L. monocytogens* has good adhesive traits important for colonization and biofilm formation.

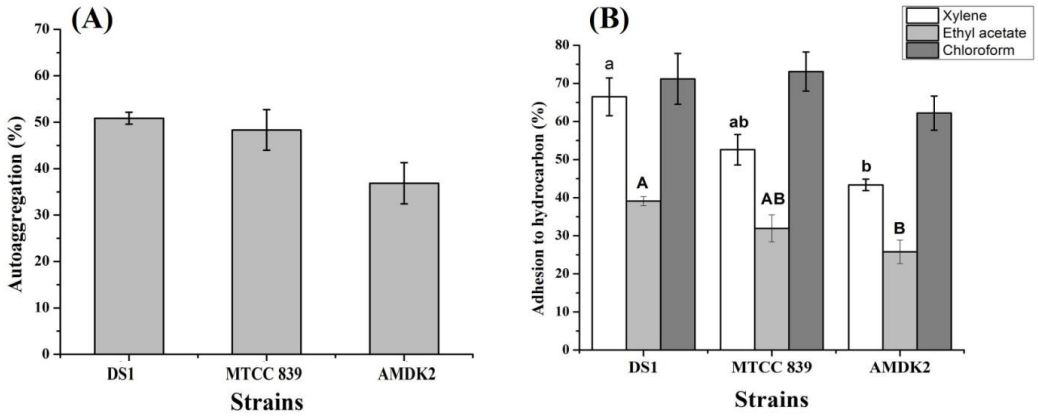


Fig. 4.7. (A) Autoaggregation abilities of probiotic and pathogens, 5(B) Bacterial adhesion to hydrocarbon assay; different letters signify statistical difference (P<0.05) as calculated by Tukey's Multiple Comparison test, OriginPro 8.5. Abbreviations: 839- *L. monocytogenes* MTCC 839, AMDK2- *L. monocytogenes* strain AMDK2, DS1- *Pediococcus pentosaceus* DS1

4.4.6. Probiotic mediated biofilm inhibition assay

Biofilm inhibition by the three different methods, i.e. competition, exclusion and displacement were compared in Fig. 4.8.

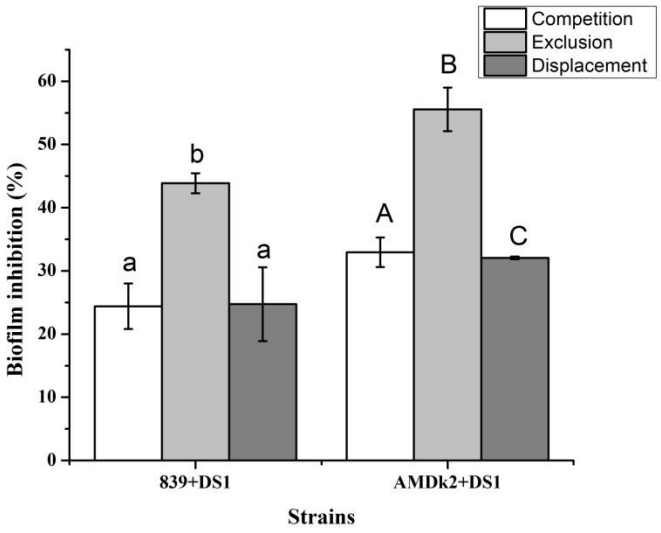


Fig. 4.8. Probiotic- mediated competition, exclusion and displacement of *L. monocytogenes* strains, different letters signify statistical difference (P<0.05) as calculated by Tukey's Multiple Comparison test, OriginPro 8.5. Abbreviations: 839- *L. monocytogenes* MTCC 839, AMDK2- *L. monocytogenes* strain AMDK2, DS1- *Pediococcus pentosaceus* DS1

.During competition assay, probiotic strains compete with pathogens for the adhesion sites. The adhesion of the strain *L. monocytogenes* AMDK2 strain reduced maximally

by 32.95 ± 2.34% after coculture. Exclusion signifies the inhibition of biofilm formation due to the presence of pre- formed biofilm and the production of antimicrobial compounds by the probiotic bacteria. When pathogens were added to the wells containing pre-grown probiotic cells, biofilm formation by the pathogens was found to be decreasing, which could be beneficial for the control of pathogens [35]. In case of *L. monocytogenes* AMDK2, maximum biofilm exclusion (55.54 ± 3.44%) was observed. The values for displacements were found to be statistically indifferent from those of the competition experiment ($P<0.05$). *L. monocytogenes* AMDK2 was found to be maximally displaced by the probiotic bacteria (32.05 ± 0.21%). Among all the biofilm inhibition tests, values of the exclusion were significantly higher than other tests (Fig. 4.8).

4.4.7. Scanning electron microscopic observations of biofilm inhibition

The MIC values of the lyophilized cell free supernatant were found to be 50 mg/ml and 12.5 mg/ml against *L. monocytogenes* MTCC 839 and *L. monocytogenes* AMDK2 respectively. When incubated with 50% MIC values, i.e. 25 mg/ml and 6.25 mg/ml respectively for *L. monocytogenes* MTCC 839 and *L. monocytogenes* AMDK2, a decrease in adhesion of bacterial cells was observed (Fig. 4.9).

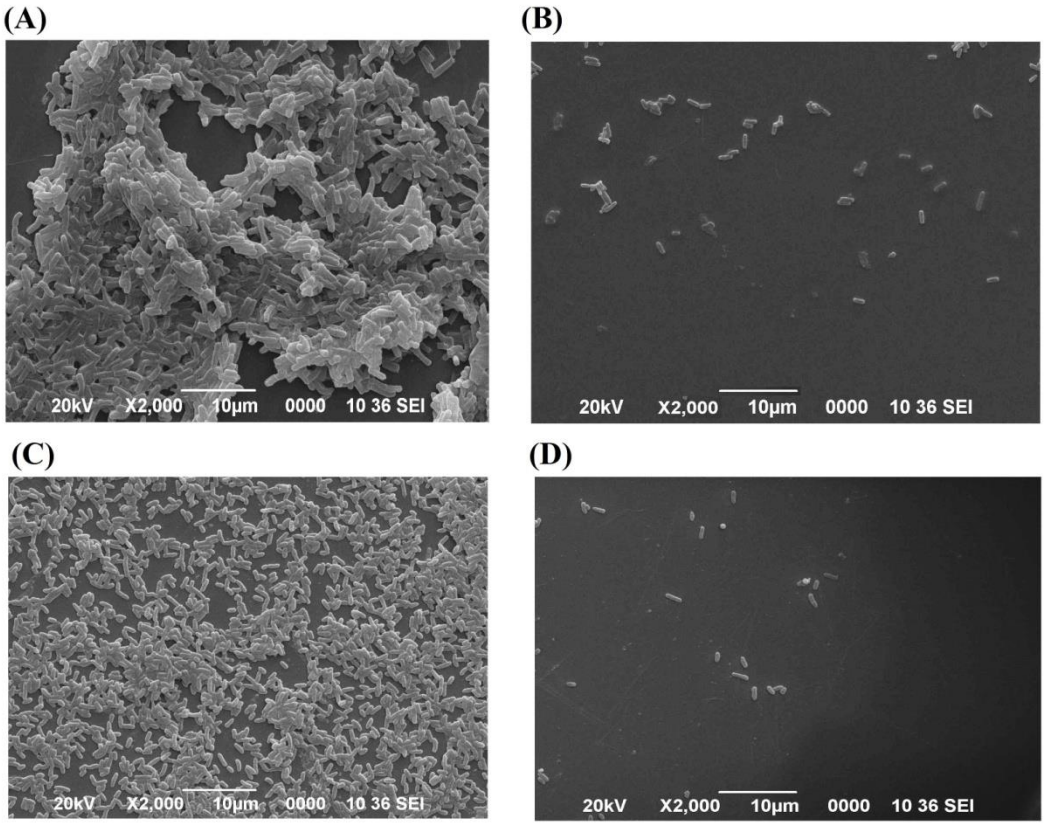


Fig. 4.9. Scanning electron micrograph (SEM) of biofilm formed by *L. monocytogenes* MTCC 839 and *L. monocytogenes* AMDK2, (A) MTCC 839 control, (B) MTCC 839 treated, (C) AMDK2 control and (D) AMDK2 treated.

4.4.8. Inhibition of *L. monocyteogenes* Caco-2 cell adhesion and invasion by *P. pentosaceus* DS1

Caco-2 cell line is the most commonly used model for studying adhesion of probiotics and inhibition of pathogen adhesion and invasion [36]. Invasion, which is a prime mode of virulence transfer of *Listeria* is mediated through surface protein Internalin A (*Inl*A) [37]. As shown in the fig. 4.10 the adhesions and invasions of the control groups were found to be significantly higher than the probiotic- treated group ($P<0.05$). Maximum decrease in adhesion was found in case of *L. monocytogenes* AMDK2 (91.8%), whereas maximum decrease in invasion was in case of *L. monocytogenes* MTCC 839 (52.9%).

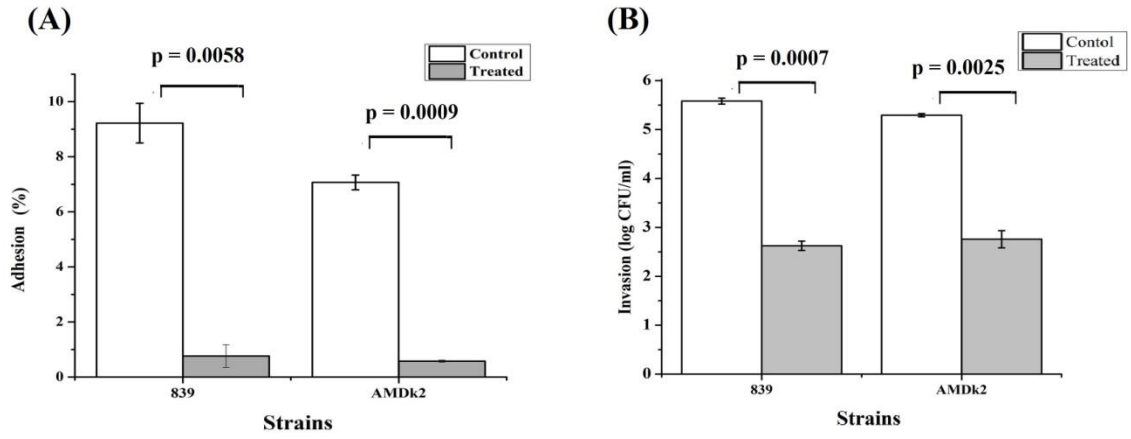


Fig. 4.10. (A) Adhesion and (B) invasion inhibition of *L. monocytogenes,* student's t-test, unpaired, OriginPro 8.5. Abbreviations: 839- *L. monocytogenes* MTCC 839, AMDK2- *L. monocytogenes* strain AMDK2. *P* <0.05 signifies statistical difference.

4.4.9. Antagonistic effect of *P. pentosaceus* DS1 against *L. monocytogenes* in milk

Fig. 4.11A and 4.11B depicts the growth curves of the pure (control) and the probiotic- treated cultures of *L. monocytogenes* 839 and AMDK2 respectively. The pure cultures of *L. monocytogenes* 839 and AMDk2 showed growth rates (μ_{max}) 0.21 and 0.25 h^{-1} respectively, with maximum counts (N_{max}) 8.28 and 8.15 log CFU/ml respectively (table 4.2). Lag phases for both the strains were ~4.5 h. However, in case of coculture experiments with *P. pentosaceus* DS1, it was found that the growth rates of the *Listeria* strains decreased considerably; maximum decrease was found in case of AMDK2, where growth rate decreased to -0.04 h^{-1}. In acidified milk, growth parameters were found to be similar to the controls. Similarly, AMDK2 showed 5.62 log units reduction in the maximum cell count after the experiment. pH of the coculture decreased below 4.

Inhibition of one bacterial strain in a coculture may be attributed to many factors, such as the production of antimicrobial substances by one strain, changes in pH, contact inhibition etc. There are many reports which established that *Listeria monocytogenes* survive in acidic conditions prevailing in acidified milk such as yogurt, curd etc [38]. Acid fermented milks are usually acidified by the lactic acid bacteria growing in it by the production of lactic acid. In our study, milk samples acidified with lactic acid did not change the growth patterns of *L. monocytogenes* strains which substantiate the works of the previous authors. This also confirms the

action of inhibitory substances other than organic acids, in our case bacteriocin, as reported by other researchers [39, 40, 41].

Table 4.2. Estimation of microbial growth parameters

strains	n	μ_{max}	se (μ_{max})	lag	se (lag)	N_0	N_{max}	se (N_{max})	se (fit)	R^2
L. monocytogenes MTCC 839 (control)	8	0.21	0.03	4.50	2.54	2.50	8.28	0.17	0.20	0.99
L. monocytogenes MTCC 839 (acidified milk)	8	0.1989	0.02744	–	–	2.52	8.032	0.3815	0.4937	0.9525
L. monocytogenes MTCC 839 (probiotic coculture)	8	0.06	0.01	–	–	2.56	3.29	0.04	0.08	0.92
*L. monocytogenes*AMDK 2 (control)	8	0.25	0.04	4.45	2.65	2.54	8.15	0.21	0.29	0.98
*L. monocytogenes*AMDK 2 (acidified milk)	8	0.2167	0.02549	–	–	2.5	8.111	0.3335	0.4461	0.9644
L. monocytogenes AMDK2 (probiotic coculture)	8	-0.04	0.01	5.66	3.21	2.53	2.53	0.01	0.05	0.99

Symbols: n- number of experiments, μ_{max} - growth rate (h^{-1}), lag - Lag period (h), N_{max} - Maximum cell count (log CFU/ml), $N0$ - initial cell count (log CFU/ml), se- standard error.

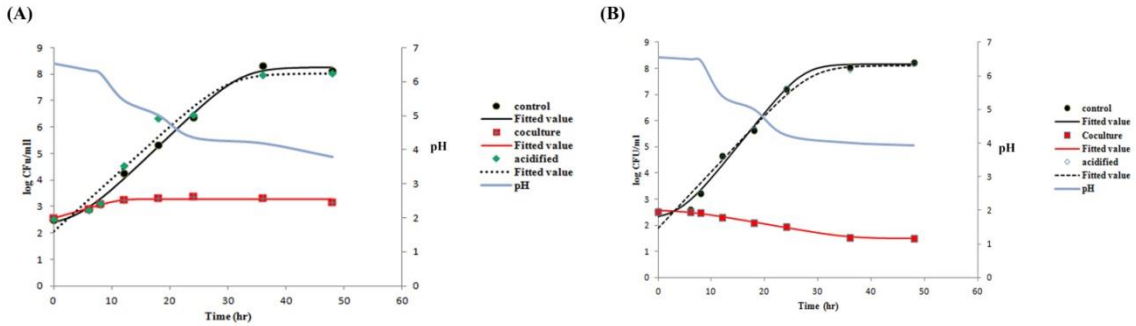


Fig. 4.11. Modelled growth curves of (A) *L. monocytogenes* MTCC 839 and (B) *L. monocytogenes* AMDK2, fitted using the DMfit curve-fitting program; version 3.5.

4.5. Conclusions

The fermented food isolate *Pediococcus pentosaceus* DS1 which showed the production of bacteriocin pediocin was found to inhibit food pathogen *Listeria monocytogenes.* The virulent properties of *L. monocytogenes* such as biofilm formation, adhesion and invasion to Caco-2 cell were minimized by the probiotic strain. Moreover, the kinetic model of inhibition of *L. monocytogenes* in coculture validates the suitability of *P.pentosaceus* DS1 to be used as a biocontrol agent for pathogen inhibition in milk products.

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