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Research Article

ASSESSMENT OF PROBIOTIC PROPERTIES OF THE STRAIN *LACTOBACILLUS PARACASEI* D6 AND ITS ANTICANDIDAL ACTIVITIES

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ABSTRACT

Objective: The objective of the study was to determine the probiotic properties of a strain *Lactobacillus paracasei* D6 (GenBank Accession No. KJ867173) and to assess its antifungal activities against certain *Candida* strains.

Methods: Antifungal activities of *Lactobacillus paracasei* D6 was tested against *Candida tropicalis* BSS7 and *C. albicans* MTCC 3017. The cell-free extract was tested against the formation of biofilm and germ tube of the aforementioned *Candida* strains. Different probiotic activities such as tolerance to simulated gastrointestinal fluid, adhesion to hydrocarbons, and Caco-2 cell line were evaluated for the strain *Lactobacillus paracasei* D6. Inhibition of *Candida* strains to Caco-2 cell line was also tested. The strains were identified using gene sequencing followed by phylogenetic tree construction.

Results: The probiotic properties of *Lactobacillus paracasei* D6 were found to be statistically comparable with a standard *Lactobacillus plantarum* MTCC 1407. The biofilm and germ tube formation patterns of the food spoilage isolate *C. tropicalis* BSS7 were found to be similar to the strain *C. albicans* MTCC 3017. Cell-free extract of *Lactobacillus paracasei* D6 exhibited minimum biofilm inhibitory concentration of 0.438 mg/ml against *C. tropicalis* BSS7, which was found to be sufficient to inhibit its germ tube formation. The adhesion of *C. tropicalis* BSS7 to the epithelial Caco-2 cell line was also significantly reduced by the antifungal metabolites.

Conclusion: This work gives insight on possible virulent nature of the food isolate C. tropicalis BSS7. Exometabolites produced by L. paracasei D6 were able to inhibit growth, biofilm and germ tube formation of both the strains. With this work, the authors envisage the beginning of an alternative strategy for anticandidal therapy.

Keywords: Biofilms, Fermented foods, Food safety, *Lactobacillus*, Probiotic.

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INTRODUCTION

Food spoilage is detrimental to the food industry that significantly affects the cost and availability of food [1]. *Candida tropicalis* is responsible for the spoilage of fruit juices (Souza *et al*., 2007) and acts as a biofouling agent in fruit juice processing plants [2].

Different species of *Candida* have been investigated for their biofilm formation abilities on various biotic and abiotic surfaces. Both biofilm and germ tube formation are very important virulence factors expressed by different *Candida* sp. isolated from clinical specimens. However, *Candida* sp. isolated from spoiled foodstuffs has never been investigated for the abovementioned factors. There are few reports where it was established that there is a homology between foodborne yeasts and their clinical counterparts in terms of biochemical profile and there is a chance of high-frequency plasmid DNA transfer between pathogenic and foodborne yeasts while grown together [3,4]. *Candida* showing putative virulent properties can evade phagocytosis and promote invasion of host epithelial cells [5].

The use of chemotherapeutic agents has been monitored since the past two decades, but due to the varying epidemiology of *Candida* infections and the development of antifungal resistance, replacement of conventional antifungal drugs with alternative therapeutic agents has become a necessity in the present-day scenario. It has been reported that probiotics and their exometabolites have the capacity to reduce the adhesion of *Candida albicans* to enterocytes and hence its virulence [6]. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [7]. They comprise mostly of lactic acid bacteria (LAB) and bifidobacteria that are added to foods as health beneficial adjuncts [8]. Lactic acid bacteria grow in nutrient-rich environments and as a result of fermentation, they produce different substances having biopreservative properties, as a result of which they are used for the preparation of fermented foods with longer shelf-life.

Different fermented bamboo shoot (FBS) products are consumed by the ethnic people living in the biodiversity-rich northeast region of India including Arunachal Pradesh which provide an optimum climate for the growth of many edible bamboo species. "Ekung" is such a product which is prepared by the Nyishi community of Arunachal Pradesh that employs their unique methods of fermentation [9]. The presence of LAB in FBS was previously reported [10]. However, health-beneficial effects of LAB present in these products are not yet completely understood. The present study deals with the antifungal and probiotic of attribute *Lactobacillus paracasei* D6 and its exometabolites as well as reduction of some of the most important virulent factors of *Candida* isolates obtained from spoiled foodstuff.

METHODS

Isolation of microbial strains and culture conditions for different strains used

The strain BSS7 was isolated from spoiled commercial orange juice and was routinely grown in potato dextrose agar (PDA) at 28°C.

Fermented bamboo shoots (ekung) were collected aseptically in sterile containers and stored at 4°C. One gram of sample was blended with sterile 0.85% (w/v) saline solution using mortar and pestle under aseptic conditions and 10-fold serial dilution was performed. Different dilutions were spread on de Man, Rogosa, and Sharpe (MRS) agar (HiMedia Labs, Mumbai) and incubated at 37°C under anaerobic conditions for 24 h. Gram-positive and catalase-negative strains [11] were selected for further evaluation.

Candida albicans MTCC 3017 and *Lactobacillus plantarum* MTCC 1407 were procured from Microbial Type Culture Collection (MTTC), Chandigarh, India. Culture media used for the growth of *Candida albicans* MTCC 3017 was PDA and growth temperature was 28°C. Similarly, *Lactobacillus plantarum* MTCC 1407 which was used as a standard probiotic strain was grown in MRS agar at 37°C under anaerobic conditions for 24 h. Both strains were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh.

Molecular identification

The isolate D6 was identified by 16S rDNA gene sequencing followed by phylogenetic tree construction. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for the amplification of 16S rRNA gene sequence [12].

For the identification of the strain, BSS7 sequencing of ITS1-5.8S rDNA intergenic region was done using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [13]. Sequencing of D1/D2 region of 26S rRNA was also done using the primers: NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTC AAGACGG-3′) [14].

Amplification of the 16S rRNA gene was done using a previously reported protocol developed in our laboratory [15]. Amplification parameters for the ITS1-5.8S rDNA intergenic region and the D1/D2 region consisted of an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, primer annealing for 30 s at 58°C, elongation for 1 min at 72°C, and final extension of 10 min at 72°C for 1 cycle. The amplified PCR products were purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystems, 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 [16-18].

Extraction of antifungal metabolites (AFMs) from the spent broth of the strain D6

A method reported by Rizzo [19] was used to extract antifungal metabolites (AFMs) from cell-free supernatant of D6 with some modifications. Cells were separated from the spent broth by centrifugation at 3000 g for 15 min, and filter sterilized (0.45 µm pore size; Millipore). Ten milliliters of the spent broth culture filtrate were taken and 4 g of NaCl and 2 ml of 50% H_2SO_4 were added. This solution was then extracted with 5 ml of diethyl ether and concentrated. Uninoculated MRS broth was used as a control.

Antifungal assays

For the determination of antifungal activity of the strain, D6 agar overlay method reported by Magnusson and Schnurer [20] was employed. D6 was streaked on to MRS agar plate as two 3 cm long lines and incubated at 37°C for 3 days. The plate was overlaid with potato dextrose agar (PDA) containing 10^4 *Candida* cells per ml and incubated at 30°C.

Minimum inhibitory concentration (MIC) values for the AFMs were determined according to a poison food technique [21] with some modifications. AFMs were mixed with molten Czapek Dox Agar (CDA) at various concentrations and *Candida* was spotted onto the agar surface. Minimum concentration of AFM that resulted in no visible mold growth after 72 h of incubation would be considered as MIC. In a different set of experiment, susceptibility toward amphotericin B, a standard antifungal drug was also evaluated using the same protocol.

Antibiofilm assay and inhibition of germ tube formation

Bacterial cells were diluted with phosphate-buffered saline (PBS, pH 7.4) to 10^7 cfu ml^{-1} and an aliquot of 20 µl was transferred to the wells of a 96-well microtiter plate containing 180 µl of potato dextrose broth (PDB) and allowed to grow for 48 h at 28°C. Different concentrations of AFMs (µg ml^{-1}) were previously added to PDB and PDB without AFM was used as control. Following incubation, wells were rinsed three times with sterile deionized water and the plates were air-dried for 45 min, and then, each well was stained with 200 µl of 0.1% (w/v) crystal violet for 30 min [22]. Crystal violate bound cells were solubilized by 100% (v/v) ethanol and absorbance was taken at 570 nm. Biofilm inhibition was calculated as

Biofilm inhibition=[(O.D.control − O.D.test)/O.D.control]×100

Minimum biofilm inhibitory concentration (BIC_{50}) was defined by the minimum concentration that resulted in 50% inhibition of biofilm formation [23].

For the visualization of germ tube formation, *Candida* strains were grown in spider medium (1% nutrient broth, 0.4% potassium phosphate, and 2% mannitol) containing 10% (w/v) fetal bovine serum (FBS) with or without the presence of BIC_{50} of AFM on 24-well microtiter plates. Plates were incubated at 37°C for 36 h [24]. Morphological transition of rounded cells of *Candida* into germ tube bearing structures was observed under light microscope at ×40 .

Probiotic characterization of the strain D6

Acid and bile tolerance

Acid and bile tolerance was performed as reported earlier [25,26]. For acid tolerance overnight bacterial culture was inoculated in to MRS broth adjusted to pH 2 and 3.5 with 3M HCl. 0.3%, 0.5% and 1.5% (w/v) bile salts (HiMedia) were added to MRS broth for testing bile tolerance. In both cases MRS broth (pH 6.4) was used as control. All samples were incubated at 37°C. After 0, 3 and 6 h, aliquots were drawn from each sample and plated on MRS agar plates for the determination of viable count.

Microbial adhesion to hydrocarbons (MAT) assay

This assay was performed following a method reported earlier [27] with minor modifications. Cells were harvested from overnight culture and washed twice with PBS, pH 7.2. Cell count was adjusted approximately to 10^9 cfu ml^{-1} and $O.D._{600}$ was measured. Now equal volume of cell suspension was mixed with n-hexadecane, chloroform and ethyl acetate separately and mixed properly by vortexing for 2 min. The two phases were allowed to separate for 1 h and the aqueous layer was gently pipetted out and $O.D._{600}$ was measured. Cell surface hydrophobicity was calculated according to the formula:

$$Hydrophobicity(\%) = \frac{A0 - A1}{A0} \times 100$$

Where, A_0 = $O.D._{600}$ at 0 h and A_1 = O.D.600 at 1 h.

Caco-2 cell culture and adhesion assay

The human colon adenocarcinoma (Caco-2) cell line was procured from National Centre for Cell Science (NCCS), Pune, and grown in minimum essential medium (MEM, St. Louis, MO, USA) supplemented with 10% (w/v) fetal bovine serum (Invitrogen, Gibco), 1% (v/v) non-essential amino acids (Invitrogen, Gibco), and 50 µg/ml gentamicin (Invitrogen, Gibco). The cells were routinely grown at 37°C in a humidified atmosphere of CO_2 (5%) until confluent growth was obtained.

The adhesion assay was carried out according to a previously reported protocol [28] with some modifications. Briefly, Caco-2 cells were grown in 6-well microtiter plates supplemented with MEM media and after confluent growth, *Lactobacillus* culture (at 1 × 10^9 cfu) suspended in 1 ml MEM medium (without serum and antibiotics) was added and incubated for 30 min. *Lactobacillus* adherence to Caco-2 cell was expressed as cfu/ml which was determined by plating on MRS agar plates.

Inhibition of adhesion of *Candida* strains to Caco-2 cell line by AFM

Caco-2 cells were seeded on a 96-well tissue culture plate at a concentration of 2.0–2.5×10^4 cells per well. Following confluence, cells were washed with PBS and replenished with fresh MEM medium supplemented with 2% FBS (without antibiotics). Freshly grown *Candida* cells were harvested by centrifugation and washed with PBS. For the adhesion assay, experimental set up consisted of (1) pure Caco-2 cell, (2) Caco-2 cells+BSS7 (control), (3) Caco-2 cells+ MTCC 3017 (control), (4) Caco-2 cells+BSS7+BIC_{50} of AFM (test), and (5) Caco-2 cells+MTCC 3017+ IC_{50} AFM (test) in separate wells. All wells contained Candida cells at a concentration of 2.0×10^6 cfu ml^{-1}, except for the wells containing pure Caco-2 cells which were not inoculated with *Candida*. Adhesion experiment was performed for 3 h at 37°C followed by washing with PBS to remove the unattached cells and fixing with 4% (w/v) *p*-formaldehyde. Cells were visualized under light microscope (EVOS FL cell imaging system, Thermo Fisher) under ×20. For the quantitative assessment of adhesion, cells were stained with 0.5% (w/v) crystal violet and absorbance was taken at 595 nm [29].

RESULTS AND DISCUSSION

Isolation and identification of strains

Based on the color formation on chromogenic medium, the strain BSS7 was presumptively identified as *Candida tropicalis*. Molecular typing of the strain is shown in Fig. 1. In this study, sequencing of both the ITS1-5.8S-ITS2 region and D1/D2 region of 26S rDNA reveals that the sequences have maximum identity to *Candida tropicalis*. After submission to NCBI GenBank, the sequences received accession numbers KT387284 and KT387283, respectively.

The Gram-positive and catalase-negative strain D6 were identified by 16S rDNA sequencing and the sequence was used to construct the phylogenetic tree. The BLAST analysis showed 99% similarity with the 16S rDNA sequence of *Lactobacillus paracasei* strain RU4-1 16S. This sequence received an accession number KJ867173.

The virulence patterns shown by the pathogens have profoundly changed during the past few decades. For instance, the non-albicans *Candida* spp. once considered as non-pathogenic contaminating agents have emerged as potential pathogens [30]. *Candida tropicalis* is a widespread yeast species and there is a dearth of knowledge regarding the virulence attributes of *Candida* sp. isolated from non-clinical sources. In our study, the identity of the strain *C. tropicalis* BSS7 was confirmed using 26S rDNA sequencing which is considered as the most consensus approach for the identification of yeasts [31].

Antifungal assays

Antifungal activity on agar overlay plates is shown in Fig. 2a and b for *C. tropicalis* BSS7 and *C. albicans* MTCC 3017, respectively. Antifungal activity was confirmed by the production of halos around streaks. The strain *L. paracasei* D6 that is producing antimicrobial substance led to the inhibition of *Candida* strains around the streaks. The minimum inhibitory concentration (MIC) of the AFM was found to be 1.75 mg ml^{-1} for *C. tropicalis* BSS7 and 3.5 mg ml^{-1} for *C. albicans* MTCC 3017, respectively (Fig. 2c and d). Both *C. tropicalis* BSS7 and *C. albicans* MTCC 3017 were also found to be susceptible to the antifungal drug amphotericin B and MIC values were found to be 0.195 µg ml^{-1} and 0.390 µg ml^{-1}, respectively.

The use of probiotics and probiotic-derived products as anti-biofouling agents has gained immense attention in food industries. Many lactic acid bacteria can inhibit the growth of yeasts and molds due to the production of acids, volatile fatty acids, cyclic dipeptides, diacetyl, phenyllactic acid, etc. [20]. In our study, the MIC values of AFMs were comparable to the MIC of a previously published report [32] reported by Nyanzi *et al*. (2014).

Antibiofilm assay and visualization of hyphal transition inhibition

The minimum concentration for biofilm inhibition (BIC_{50}) of AFM against *C. tropicalis* and *C. albicans* was calculated as 0.438 mg ml^{-1} and 0.875 mg ml^{-1}, respectively, as shown in Fig. 3a. These concentrations were found to be lower than the minimum inhibitory concentrations (MIC) for both the species. The exometabolites of lactic acid bacteria are shown to exhibit anticandidal effects that inhibit the adhesion of *Candida albicans* (Ceresa *et al*., 2015). Qualitative observation under light microscope (×20) also confirms that BIC_{50} concentrations loosen biofilm cells resulting in dispersed growth (Fig. 3d and e) as compared to the control (Fig. 3b and c). BIC_{50} concentration of AFM was found to be sufficient for the germ tube formation in *Candida*, as shown in Fig. 4.

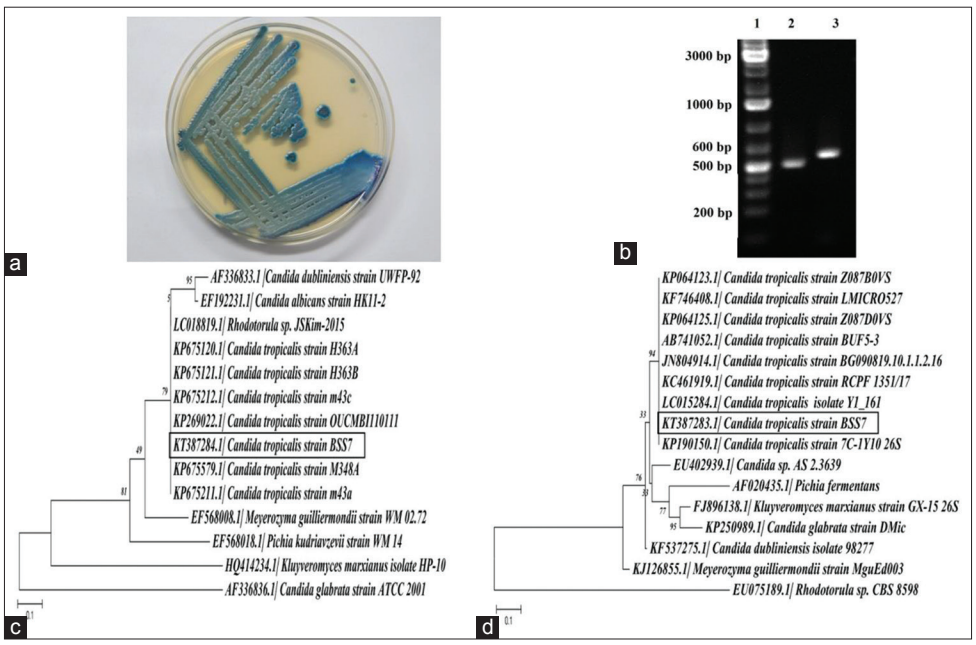

Fig. 1: Identification of the strain BSS7. (a) Colony morphology in HiCrome *Candida* differential agar. (b) Molecular typing using internal transcribed spacer primer (Lane 2) and 26S rRNA gene D1/D2 primer (Lane 3); Lane 1 indicates GeneRuler 1 Kb plus DNA ladder (Fermentas). Phylogenetic tree showing the position of BSS7 with closely related species based on (c) 5.8S ITS rRNA and (d) D1/D2 26S rRNA sequences. Bootstrap values (1000 replicates) are indicated at branch nodes

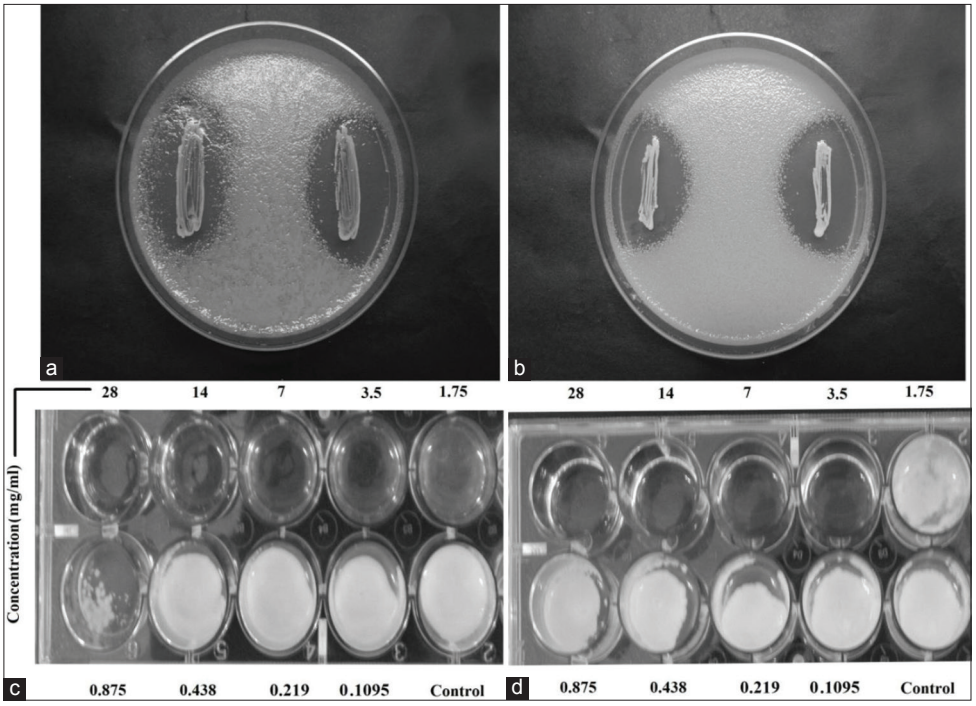

Fig. 2: Antifungal activities of *L. paracasei* D6 against (a) *C. tropicalis* BSS7 and (b) *C. albicans* MTCC 3017 visualized as halos in agar overlay method. MIC values of *Lactobacillus* metabolites against (c) *C. tropicalis* BSS7 and (d) *C. albicans* MTCC 3017, respectively

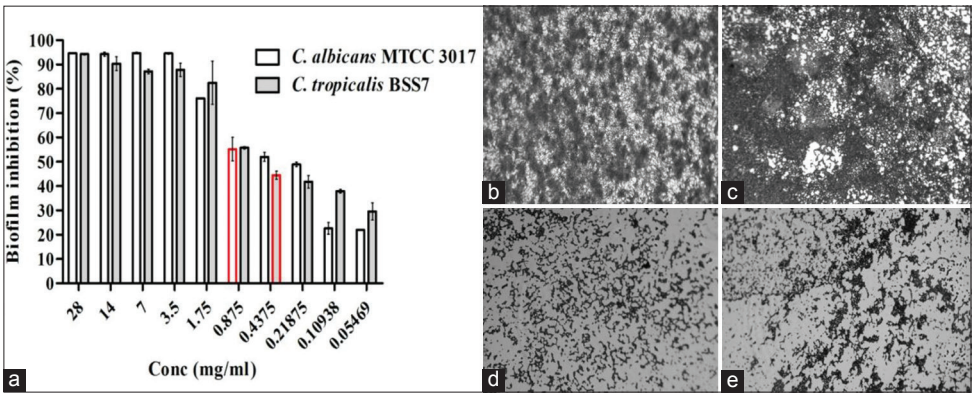

Fig. 3: Biofilm inhibition assay. (a) Biofilm inhibition by various concentrations of *Lactobacillus* metabolites; bars with red borders indicate BIC_{50}. Biofilm formation by *C. tropicalis* BSS7 on microtiter plates (b) without treatment and (d) treatment with BIC_{50}. Biofilm formation by *C. albicans* MTCC 3017 (c) without treatment and (e) treatment with BIC_{50} as observed under light microscope (×20)

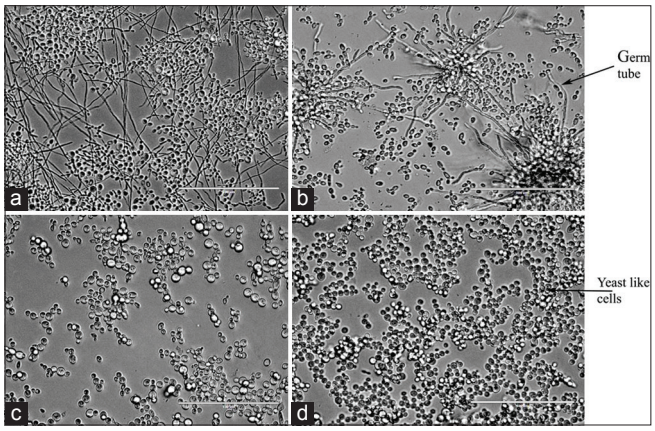

Fig. 4: Germ tube inhibition assay. (a) *C. tropicalis* BSS7 control, (b) *Candida albicans* MTCC 3017 control, (c) *C. tropicalis* BSS7 treated with BIC_{50}, and (d) *C. albicans* MTCC 3017 treated with BIC_{50}, as observed under light microscope (×40)

Table 1: Acid tolerance of *Lactobacillus plantarum* MTCC 1407 and *L. paracasei* D6

Time (h)	Strains	pH 2	pH 3.5
0	MTCC 1407	7.87±0.090	7.74±0.302
	D6	7.94±0.070	7.92±0.060
3	MTCC 1407	7.08±0.166*	8.08±0.176
	D6	6.59±0.129*	8.06±0.090
6	MTCC 1407	5.79±0.083**	7.43±0.040*
	D6	6.40±0.184**	7.85±0.320*

The transition of *Candida* cells from yeast like morphology to germ tube and hypha bearing structure is essential for biofilm formation because the hyphal cell wall contains proteins which aid in adhesion to different surfaces and ultimately biofilm formation [33]. Therefore, it can be inferred that biofilm inhibition mechanism predominantly lies in yeast-hyphal transition inhibition. Inhibition of *Candida* germ tube development by compounds isolated from

cell-free supernatant of lactic acid bacteria has been previously reported [34].

Probiotic characterization

When exposed to acidic conditions, the viability of both *L. paracasei* D6 and *L. plantarum* MTCC 1407 was found to be decreasing by only 1 log unit (Table 1) after incubation for 3 h at pH 2. However, growth at pH 3.5 was found to be statistically comparable to that of control. From Table 2, we can observe that for the strain, D6 growth did not change significantly after exposure to different concentrations of bile. However, a decreasing growth pattern was observed in case of MTCC 1407.

As shown in Table 3, both D6 and MTCC 1407 showed more adherences toward chloroform followed by n-hexadecane. It was found that *L. paracasei* D6 was the most hydrophobic strain (32.8%). Affinity toward ethyl acetate was found to be the least in case of both the strains (<20%).

In our study, maximum adhesion to Caco-2 cell line was found to be in case of the strain D6 which is more than MTCC 1407 (Table 2). This can be correlated with the results of MAT assay that indicated that hydrophobicity of the strain D6 is more than that of MTCC 1407.

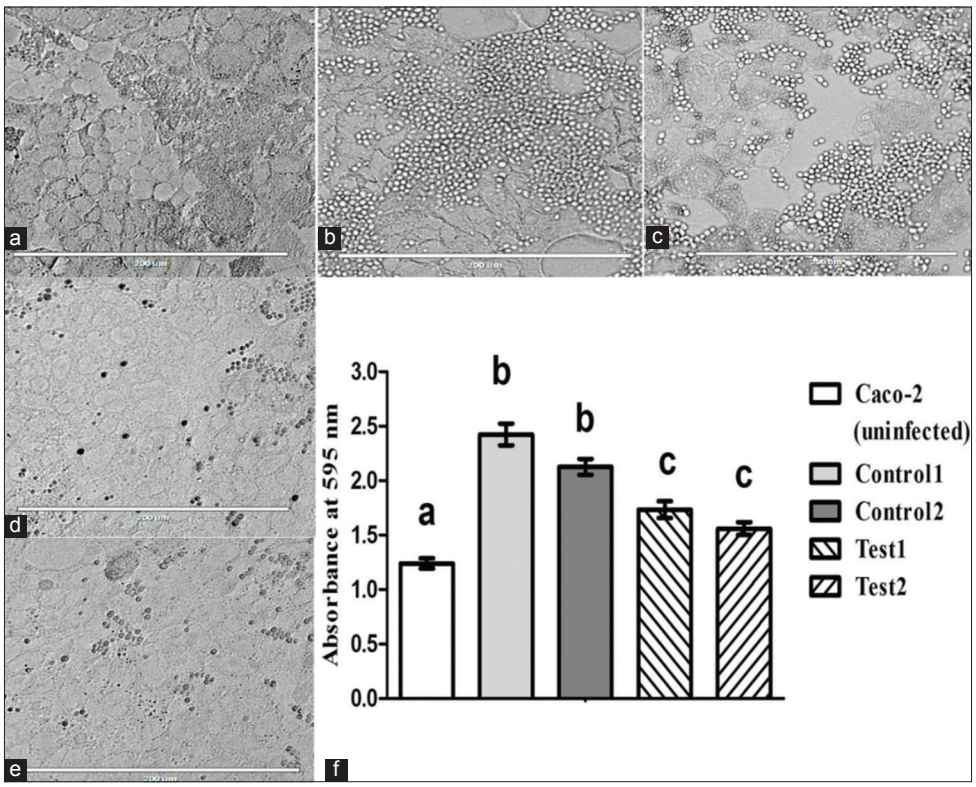

Fig. 5: *Candida* adhesion inhibition assay. (a) Uninfected Caco-2 cell, (b) Caco-2 cells + BSS7 (control 1), (c) Caco-2 cells+ MTCC 3017 (control 2), (d) Caco-2 cells + BSS7 + BIC_{50} of AFM (test 1), (e) Caco-2 cells + MTCC 3017 + BIC_{50} AFM (test 2), and (f) spectrophotometric adhesion inhibition assay; different letters signify statistical difference (p<0.05), estimated by Turkey's multiple comparison test, GraphPad Prism, Ver. 5.0

Table 2: Bile tolerance of *Lactobacillus plantarum* MTCC 1407 and *L. paracasei* D6

Time (h)	Strains	Bile tolerance			
		Control	0.3% (w/v)	0.5 (w/v)	1.5 (w/v)
0	MTCC 1407	7.66±0.180	7.68±0.030	7.76±0.005	7.62±0.090
	D6	7.81±0.099	7.83±0.062	7.87±0.131	7.55±0.068
3	MTCC 1407	8.15±0.098	7.25±0.007**	6.27±0.160***	5.85±0.064***
	D6	7.87±0.341	7.57±0.100	7.40±0.140	7.31±0.080
6	MTCC 1407	8.26±0.140	5.68±0.032***	4.76±0.005***	4.62±0.088***
	D6	8.03±0.014	7.78±0.180	7.68±0.120	7.62±0.080

Statistical differences were estimated by Student's t-test (non-parametric), GraphPad Prism, ver. 5.0. Values are represented as mean ± SD., n=3. Student's t-test, GraphPad Prism, ver. 5.0. *p<0.05, **p<0.01, ***p<0.001

Table 3: Adhesion of *Lactobacillus plantarum* MTCC 1407 and *L. paracasei* D6 toward hydrocarbons and Caco-2 cell line

Strains	Adhesion to hydrocarbons			Adhesion to Caco-2 cell line
	Ethyl acetate (%)	Chloroform (%)	n-Hexadecane (%)	
MTCC 1407	19.54±8.026	49.47±3.470	30.37±7.261	8.63±0.730
D6	17.66±1.930	47.13±5.835	32.38±2.350	9.93±0.930

Resistance to acid and bile salts is considered as prerequisite traits for probiotic evaluation [35]. Affinity of probiotic cell surface toward hydrocarbons is considered as a key factor for adhesion. This plays a crucial role in intestinal colonization and inhibition of pathogen access through steric interactions [36]. Caco-2 cell line has been used as an intestinal cell model for experiments such as probiotic adhesion and exclusion of pathogenic bacteria since this cell line shows characteristics of mature enterocytes and forms functional brush border microvilli and apical hydrolases [37].

Inhibition of adhesion of *Candida* strains to Caco-2 cell line

Fig. 5 illustrates *Candida* adhesion to Caco-2 cells in the presence or absence of AFM. It was found that crystal violet absorption by pure Caco-2 cells was significantly lower ($p<0.05$) than the control wells containing *Candida*-infected Caco-2 cells (Fig. 5f). Adhesion capacities of BSS7 and *C. albicans* were not significantly different. However, in the test wells, there was significant decrease in adhesion compared to the respective control groups suggesting possible anti-adhesive properties of AFM which inhibited the adhesion of both *Candida* strains.

The propagation of *Candida albicans* was largely due to the translocation of fungal cells from the gut to the bloodstream and initial propagation is mediated by the adhesion of *Candida* cells to the enterocytes [38]. Therefore, adhesion inhibition of *Candida* to epithelial cell may be considered as the first step toward minimizing *Candida*-related infections.

CONCLUSIONS

Food safety is a major concern of this present era due to the emergence of pathogenic microorganisms with unprecedented virulent properties and antimicrobial resistance. This work stresses on possible virulent nature of food isolate *C. tropicalis* BSS7 that mimics biofilm forming strain *C. albicans* MTCC 3017. Exometabolites produced by *L. paracasei* D6 were able to inhibit growth, biofilm, and germ tube formation of both the strains which give an insight into an alternative strategy for anticandidal therapy.

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AUTHORS' CONTRIBUTIONS

Both the authors equally contributed with this research work.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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