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In vitro investigation on probiotic, anti-*Candida*, and antibiofilm properties of *Lactobacillus pentosus* strain LAP1

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ABSTRACT

Objective: To investigate the probiotic characteristics, anti-*Candida* activity, and antibiofilm attributes of *Hentak* derived *Lactobacillus pentosus* strain LAP1.

Design: The probiotic properties of strain LAP1 was depicted by adapting standard protocols. The anti-*Candida* and antibiofilm properties of isolate were determined using agar well diffusion assay and ELISA reader test, respectively. The time-kill assay was performed using viable colony count assay. Further, the co-aggregation property of strain LAP1 was determined based on standard methodology.

Results: Strain LAP1 exhibited not only tolerance to acidic pH but also showed resistivity ($P \leq 0.05$) to simulated gastric juice exposure. Similarly, the strain was able to tolerate bile salt, showed hyperproteolytic activity, and also depicted susceptibility to most of the antibiotics tested. Auto-aggregation phenomenon (37.5–60%), hydrophobicity nature (42.85%), and survival potentiality of strain LAP1 under freeze-dried condition (9.0 ± 0.01 log CFU/ml) made the isolate a promising probiotic candidate. Cell-free neutralized supernatant (CFNS) of strain LAP1 exhibited potent antifungal activities against *C. albicans*, *C. tropicalis*, and *C. krusei* with arbitrary unit of 150 ± 4.34, 200 ± 5.21, and 130 ± 5.13 AU/ml, respectively and depicted remarkable reduction in the biofilm formation of respective *Candida* sp. in a concentration dependent manner. Moreover, time-kill assay data provided the growth inhibition of all *Candida* sp. in a time dependent manner. Additionally, strain LAP1 revealed significant co-aggregate percentage with *C. albicans*, *C. tropicalis*, and *C. krusei*.

Conclusions: *L. pentosus* strain LAP1 exhibited a good probiotic characteristics, potent anti-*Candida* activity, and significant antibiofilm property that could be undoubtedly recommended for its vast applications not only in food industries but also as biotherapeutic agent against *Candida* infections in pharmaceutical industries.

1. Introduction

Probiotics are non-pathogenic live microorganisms which when ingested in adequate quantity exert health benefit on their host (Nyanzi, Jooste, Cameron, & Witthuhn, 2013). In recent years, Lactic acid bacteria (LAB), especially *Lactobacillus* sp. have been exploited extensively due to their probiotic characteristics which are generally recognized as safe (GRAS). Lactobacilli have been recognized as commensal flora that functions to combat pathogenic bacteria by releasing antimicrobial metabolites. They are found as normal flora in the diverse sites of human body viz. oral cavity, gastrointestinal tract, and female urogenital tract. The site-specific isolation of lactobacilli is very important due to the dependent nature of probiotic efficacy on the species and the specific niche targeted. It is noteworthy that the biopotency of lactobacilli is strain specific (Moal & Servin, 2014); therefore, new investigations are beginning to assess the probiotic traits of lactobacilli from diversified origins such as oral cavities, human vagina, and other exogenous sources.

Health benefits of *Lactobacillus* sp. include prevention and treatment of various types of bacterial and fungal diseases. Few of the potential benefits of LAB include prevention of colon cancer, lactose intolerance, diarrhoea, hypercholesterolaemia, and other intestinal infections

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(Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Holzapfel & Schillinger, 2002; Rousseau, Lepargneur, Roques, Remaud-Simeon, & Paul, 2005). Recently, *Lactobacillus* sp. of various origins had been reported to exhibit antifungal activities that could be applied as biopreservatives (Coloretti et al., 2007; El-Mabrok, Hassan, Mokhtar, & Hussin, 2013; Oliveira, Zannini & Arendt, 2014). Organic acids, hydrogen peroxides, diacetyl and/or proteinaceous bioactive compounds are one of the major constituents of lactobacilli that are responsible for the broad spectrum antifungal potentialities.

Among fungal infections, Candidiasis is one of the most common yeast infections which are caused by different species of *Candida*, especially, *Candida albicans, Candida tropicalis*, and *Candida parapsilosis* (Papon, Courdavault, Clastre, & Richard, 2013). At present, wide spread of immune-compromised infections and over doses of various anti-*Candida* drugs resulted into the emergence of drug resistance *Candida* sp. Four different mechanisms are known to be involved in the development of drug resistance *Candida* sp. (i) efflux pumps induction, leading to the reduction of azole concentration inside the cell, (ii) bypass pathways development, (iii) up regulation of *CDR* or *MDR* genes encoding efflux pumps, and (iv) ERG11 gene mutation (Peman, Canton, & Espinel-Ingroff, 2009). Biofilm formation is one of the most important virulence factors for *Candida* sp. which confers resistivity towards antifungal agents by blocking their penetration through the matrix and protecting cells from host immune responses (Ramage, Saville, Thomas, & Lopez-Ribot, 2005). Biofilms formed by *Candida* sp. have high resistance to antifungal therapy that attributes this fungus difficult to diagnose and treat. *Candida* sp. has ability to hedge host defences and form biofilm not only on host tissue but also on medical devices. LAB supernatants contain bioactive antagonistic components which can avoid or reduce adhesion property of pathogens to medical devices (Rodrigues, Van der Mei, Teixeira, & Oliveira, 2004).

Several types of *Candida* sp. are resistant to numerous non-natural antifungal agents and they are not easily killed by normal antifungal agents used for health therapy (Bulgasem, Lani, Hassan, Yusoff, & Fnaish, 2016). Various bioactive components such as acids, hydrogen peroxide, diacetyl, and bacteriocins produced by LAB have been considered promising antifungal agents. The role of fermented food associated LAB as antifungals has received little attention and their mechanism of inhibitory against fungi is not fully understood. The present scenario of limited anti-fungal drugs, emergence of drug-resistant *Candida* sp., and failure along with high cost of existing anti-*Candida* drugs show an urgency to find a novel, cost-effective and harmless approach towards the growth inhibition of *Candida* sp. In view of the GRAS status of LAB and considering their effective but less exploited role as antifungal agents, the present context was investigated to determine the probiotic, anti-*Candida*, and antibiofilm characteristics of *Lactobacillus pentosus* strain LAP1, isolated from *Hentak* (a fermented fish food product of North-East India).

2. Materials and methods

2.1. Microorganisms of interest, media, and culture conditions

L. pentosus strain LAP1 (Accession No: KU945826) was isolated from *Hentak* and identified (by morphological, biochemical and molecular characterization) as described earlier (Aarti, Khusro, Arasu, Agastian, & Al-Dhabi, 2016). Strain LAP1 was maintained onto MRS (de Man, Ragosa and Sharpe) agar medium (g/l – dextrose 20.0, proteose peptone 10.0, beef extract 10.0, yeast extract 5.0, polysorbate 80 1.0, sodium acetate 5.0, ammonium citrate 2.0,magnesium sulfate 0.1, manganese sulfate 0.05, dipotassium phosphate 2.0, pH 6.5, Agar 18.0) and preserved in 20% (v/v) glycerol stock at −80 °C. *C. albicans, C. tropicalis*, and *Candida krusei* collected from the Entomology Research Institute, Loyola College, Chennai, India, were used as indicator cultures for antifungal assay. All the indicator fungal cultures were grown and maintained on Potato Dextrose Agar (PDA; g/l: Potatoes 200.0, Dextrose 20.0, Agar 18.0; Distilled water 1000 ml, pH 5.6) plates at 28–30 °C for further study. All the chemicals and reagents used in the present study were procured from Himedia, India.

2.2. Probiotic characteristics of strain LAP1

2.2.1. Resistance to low pH

The potentiality of strain LAP1 to resist low pH was evaluated based on the methodology of Delgado, O'Sullivan, Fitzgerald, and Mayo (2007) with minor modifications. MRS broth media of varying pHs ranging from 3 to 6 (control- pH 6.5) was prepared and sterilized. After cooling of the media, strain LAP1 grown previously at exponential phase was inoculated into it. The broth culture was incubated at 30 °C for 90 min. After required incubation period, the pH of the medium was recorded again and the viability of the isolate was observed by the plate counting method on freshly prepared MRS agar plates.

2.2.2. Simulated gastric juice resistance assay

The tolerance characteristic of strain LAP1 to simulated gastric juice was assessed according to the method of Charteris, Kelly, Morelli, and Collins (1998a) and Charteris, Kelly, Morelli, and Collins (1998b) with some modification. Pepsin (3 mg/ml) and sodium chloride (0.5% w/v) were used for the preparation of simulated gastric juice and pHs of the solution were adjusted to 3 and 4. The overnight grown culture of strain LAP1 (30 ml) was centrifuged at 8000*g* for 10 min. Later, cells were washed with 10 ml of 50 mM K_2HPO_4. The washing step was repeated again and cells were resuspended in 3 ml of K_2HPO_4 of same molarity. After that simulated gastric juice (9 ml) of varying pH was mixed with 9.17 log CFU/ml of cell suspension and incubated at room temperature for 3 h. Hundred microliters of cell suspension were spread on the sterilized MRS agar plates at different time interval and the enumeration of total viable cells was conducted after incubating the plates at 30 °C for 48 h.

2.2.3. Determination of bile resistance

The survival rate of strain LAP1 in the presence of bile salt was determined according to the method of Vinderola and Reinheimer (2003). The bacterial suspension with about 9.17 log CFU/ml was inoculated into MRS broth supplemented with Sodium deoxycholate (0.3% w/v). The bacterial growth was observed by reading absorbance at 600 nm up to 48 h and compared with that of control (bacterial culture without Sodium deoxycholate).

2.2.4. Proteolytic property of strain LAP1

The proteolytic attribute of strain LAP1 was determined according to the methodology of Khusro (2016). One millilitre of exponentially grown strain LAP1 was centrifuged at 8000*g* for 15 min at 4 °C and the supernatant was collected. The agar plates consisting skim milk (% w/v: peptone 0.5; beef extract 0.3; skim milk 1.0, and agar 1.8) were prepared and allowed to cool aseptically. The agar media were punched with sterilized cork borer and bacterial supernatant was poured into it for the diffusion process. The plates were incubated at 30 °C for 48 h and assessed for proteolytic activity by observing clear zone of substrate degradation surrounding the wells.

2.2.5. Antibiogram assay

The susceptibility assay of strain LAP1 to different antibiotics was determined by disc diffusion method (Kirby-Bauer method; Bauer, Kirby, Sherries, & Turck, 1966). A homogeneous lawn of the exponentially phase grown isolate was prepared on MRS plates. The standard antibiotic discs (Himedia, India) such as Streptomycin (S- 10 µg), Penicillin G (P- 10 µg), Chloramphenicol (C- 30 µg), Kanamycin (K- 30 µg), Gentamicin (GEN- 10 µg), Nalidixic acid (NA- 30 µg), and Rifampicin (R- 5 µg) were placed aseptically over the agar plates and incubated undisturbed at 30 °C for 48 h. The diameter of the zone of bacterial growth inhibition was measured after required period of