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In-Vitro evaluation of probiotic effect of Lactobacillus species for the inhibition of biofilm formation by *Candida albicans*

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Abstract

Probiotic bacteria, Lactobacillus are well known to have a positive influence on the maintenance of human health by inhibiting pathogenic microorganisms. Lactic acid, bio-surfactant and bacteriocin are usually produced by Lactobacillus. *Candida albicans* as a dimorphic fungus grows both as yeast and filamentous cells. *C. albicans* is the most prevailing pathogen which causes the disease as an opportunistic pathogen in humans. The objective of this in vitro study model was to determine the effectiveness of *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei* isolated from local fruit sources against biofilm growths of *C. albicans*. Man, Rogosa and Sharpe (MRS) agar was used for isolation of bacteria. The confirmation of Lactobacillus species was carried out by microscopic as well as biochemical tests. *C. albicans* samples were collected from high-sugar mango fruit and the isolation was carried out by Sabouraud's dextrose agar (SDA). Biofilm of *C. albicans* was observed by micro-titration plate by using *C. albicans* cell suspension. The antagonistic effect of isolated *Lactobacillus* species on the biofilm produced by *C. albicans* was assessed using probiotic assay. *Lactobacillus* species induced a significant inhibition ($p<0.05$) in biofilm growths of *C. albicans*. The anti-biofilm potential of all *Lactobacillus* species was significantly ($p<0.001$) different from each other with *Lactobacillus casei* inducing maximum biofilm inhibition. The screening of probiotic characteristics and exometabolites produced by anti-candidal *Lactobacillus* species could precede efficacy studies for use these bacterial strains in cure of different candidal infections.

Keywords: Probiotics, Candida spp, Candidiasis, Lactobacillus spp, Biofilms.

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1. INTRODUCTION

Pathogenic fungal species cause over 300 different disease conditions in humans across the globe that range from mild infections to severe and chronic lethal. *Candida albicans* is a dimorphic (yeast and hyphae) fungus possessing commensal symbiotic characteristics that generally colonizes mucosal surfaces of oral

cavity, vagina and gastro-intestinal tract[1]. It is normally found in the buccal mucosa of 30 to 45% of adults. It also found in some fruits specially fruits containing high concentration of carbohydrate. The major disease manifestations caused by this pathogenic fungus include mucosal oral candidiasis, vulvo-vaginal candidiasis and systemic tissue infections[2].

The vaginal yeast infection, vulvovaginal candidiasis, is characterized most of the time by pruritis, erythema at vulvas, skin itching at areas and abnormal watery discharge from vagina of infected women[3]. This common infection affects about 75% of women population all over the world at least once in their life time and more than 5% of these affected women experience recurrent infection later on[4]. In hospitalized and immune-compromised patients, fungus infections are amongst the most frequent illnesses induced by pathogenic microorganisms[5]. Immuno-compromised persons, patients with transplants, infants born with low weight at birth, and chemotherapy patients are most vulnerable populations to infectious *Candida* diseases. In most of affected populations the infection is usually manifested in the form of bloodstream infections having a potential to disseminate into vital body organs that might include liver, heart and spleen[6].

Candida species predominantly live in host humans in the form of biofilm phase, that are structured cell cultures bound to mucosal surfaces and encapsulated within a matrix of extracellular substances of complex organic nature[7]. The biofilm formation process of *Candida* species can be divided into three different functional phase stages which include attachment, colonization and maturation phase[8]. Extracellular polymeric materials of *Candida albicans* like β- 1, 6-glucan, β-1, 3-glucan and α-mannan are amongst key polysaccharide components of biofilms. The depth of *Candida albicans* biofilm is hundred microns and it exhibits a 3-D structure[9].

The excessive use of antifungal and antimycotic drugs can cause side effects leading to resistance development in target microbial populations. Furthermore, the pattern of efficacy of these drugs is compromised owing to a dramatic increase in drug-resistant properties most of the Candida strains worldwide[10]. The possession of persister cells, initiation of efflux pump activity, and mechanical exclusion of antimicrobial drugs makes it difficult to remove biofilm[11]. Biofilm forming Candida are thousand times more resistant to various anti-fungal drugs than the single cell form[12]. These rises in resistance development in micro-organisms to traditional antifungal medications have encouraged and prompted research for alternative therapies against infections induced by pathogenic Candida species.

World Health Organization (WHO) defines that probiotic care viable micro-organisms with an ability to impart health-related benefits to host organisms when adequately administered in sufficient quantities. They are commonly found in a wide range of products including routine food items, dietary food supplements, and pharmaceuticals. Some microorganisms, such as *Bifidobacterium*, *Saccharomyces cerevisiae*, *Escherichia coli*, and Bacillus are being used as probiotics in addition to Lactobacillus[13]. Probiotics are living micro-organisms that, when ingested in adequate amounts, provide health benefits to the host in addition to supplying inherent nutrients. They are health-promoting living microorganisms that improve the intestinal microbial equilibrium and thus benefit human health. Keeping in view all these pointed, use of probiotic bacteria are suggested as a reciprocal preventive and curative treatment modality against human Candida infections[14].

Lactic acid bacteria (LAB) are a very diverse class of micro-organisms that can be found in a variety of food items as well as in animals' gastrointestinal and urogenital tracts. These microorganisms have ability to produce antimicrobial compounds including bacteriocins and bacteriocin-like growth inhibition substances. Organic acids such as lactic acid and acetic acid produced by lactic acid bacteria are important antimicrobial compounds and have been reported to possess antifungal activity. Lactobacillus bacteria produce antifungal substances such as benzoic acid, methyl hydantoin, mevalono-lactone and short-chain fatty acids. The antifungal species of *Lactobacillus* are *Lactobacillus rauteri*, *Lactobacillus rhamnosus* & *Lactobacillus casei*[15].

The exact mechanisms involved to inhibit growths of pathogenic *Candida albicans* by probiotic bacteria are unknown. It is still unclear whether these growth inhibiting effects are due to direct meddling by bacterial

cells with biofilm formation and development or due to indirect actions of exometabolites secreted by these probiotics. Keeping in view these limitations, studies are still needed to further annotate antagonistic effects of Lactobacillus strains against *C. albicans* specifically on basis of biofilm formation and hyphal filamentation potential. The blossoming of new technologies based on controlling biofilm growth of Candida spp. is envisaged as a major advancement in medicine and will have a strong influence in clinical practice and preventive medicine. Therefore, the objective of this present study to evaluate the probiotic effect of Lactobacillus species isolated from different vegetables and fruits against *Candida albicans* biofilms.

2. MATERIALS AND METHODS

2.1 Sample's collection and processing

For isolation of *Lactobacillus* spp. various fruits and vegetables samples were collected in polythene bag from different locally available shops and mini marts. For the isolation of fungus, sample of high sugar fruits (bananas and mangoes) were collected from the same shops. After collection, all the samples were transferred to the reference probiotic laboratory at the storage temperature (4 °C) in Institute of Microbiology (IOM), University of Agriculture, Faisalabad. For the isolation and broth enrichment of Lactobacillus spp. 1g of fruit and vegetable samples were dissolved in 9ml of normal saline to make the liquid form of samples.

For enrichment, 1 ml of diluted and prepared sample was taken and inoculated it in 9 ml of MRS broth. Inoculated broth tubes were incubated in an incubator for 48 hours at 37 °C. 10 sterilized test tubes were taken and labeled as $10^{-1,}$ 10^{-2} to 10^{-10}. 9ml of sterile MRS broth was taken in every labeled test tube. One ml of culture broth was added in first tube by sterilized pipette and then made 2-fold dilutions up to the 10^{th} tube. All the fruit and vegetable samples were serially diluted by same method.

2.2 Inoculation of Samples on Culture Media

Lactobacillus species were cultured on De Man Rogosa and Sharpe agar (MRS) and incubation was done for 24 hours at 37 °C. The culturing of *Candida albicans* species was performed on Sabouraud Dextrose Agar (SDA) and all petri plates were kept at room temperature (25 °C) for 48 hours. After 2 days Petri plates were observed for fungal growth

2.3 Identification of bacteria

Identification of different Lactobacillus species was done by observing macroscopic and microscopic characteristics. Fungal was observed microscopically for further identification after staining with lacto phenol cotton blue (LPCB) stain. For further confirmation different biochemical tests which includes catalase, methyl red, indole, citrate utilization, Voges Proskauer, oxidase and sugar fermentation tests were performed.

2.4 Bacterial cell harvesting

Cell harvesting is a procedure in which cells are collected from culture surface and are separated from the culture medium. Cell harvesting usually done by centrifugation technique. Rotation per minute (Rpm) of centrifugation is important because at low Rpm bacterial cells cannot be separated from the cell culture and if the speed too much high cell wall will be damage leading to destruction of the cells.

2.5 Bacterial cell suspension

PBS is an isotonic buffer often used in transportation of cells, washing of cells and making cells dilutions. It is almost isotonic to the body cells in pH, osmolarity and ion concentration. A clean glass flask was taken. 8 g of sodium chloride was added into the flask. 0.2 g of potassium chloride was added to the above solution.144 µg of disodium hydrogen phosphate was added to the solution. Then 0.245 g of Potassium dihydrogen phosphate was added. Then distilled water was added and made the total solution one litre.

The pellets containing falcon's tubes were taken. 10 ml of PBS was added with the help of micro pipette. The bacterial cell's pellets were dissolved in the PBS by vigorously shaking. The concentration of bacterial cells as 107 cells per ml was adjusted by measuring the optical density OD value at 560nm. Cell suspension was stored at 4 °C.

2.6 Preparation and preservation of Candida albicans spores' pellets

0.1 ml of tween 80 solution was taken, and it was added in 99.9ml of distilled water to make 0.1 percent solution of tween 80. Seven days old *Candida albicans* fungal culture was taken.10 ml of 0.1 percent tween 80 solution was added on the surface of 7 days old fungal culture. Then fungal culture was tightly scraped with the help of sterile scrapper for dislodging of the fungal spores. Clean sterilized glass test tubes were taken. This spore suspension was transferred into the test tubes. This cell suspension was centrifuged at the 4000 rpm for 20 minutes. After centrifugation spores' pellets were observed at the bottom of the tubes. The supernatant was discarded carefully in the sink.10ml of 50% glycerol solution was added in the test tubes containing fungal spores' pellets. Fungal spores' pellets were stored at 4 °C.

2.7 Candida albicans spores stock suspensions

Spores' pellet test tubes were taken and placed them at the room temperature for thawing. After thawing fungal spores were added to Sabourauds dextrose broth. Tube turbidity level was adjusted at 0.5 Macfarland turbidity standards.

2.8 Candida albicans biofilm formation

A sterilized 96 wells flat bottom micro titration plate was taken. 50 µl of *Candida albicans* spores' stock solution was added in row A, B and C up to the 11th wells. In the 12th wells of the three rows normal saline was added as a negative control. The micro titration plate was covered with lid. The plate was incubated for 6-7 days at room temperature. After incubation wells were washed with PBS to remove the extra spores. Biofilm was fixed with 95% ethanol. Titration plate was placed for 5 minutes to fix the biofilm. After 5 minutes wells were washed with the PBS to remove extra alcohol. After incubation and washing, biofilm was stained with lactophenol cotton blue. 50 µl of lactophenol cotton blue was added into the wells of titration plate up to 11th wells. Titration plate was placed at flat surface for proper staining. Then titration plate was again washed with PBS to remove the extra stain. OD values of each well of row A, B and C at 570nm were measured to check the biofilm formation of *Candida albicans* by spectrophotometry. The OD value was also measured of negative control well.

2.9 Fungi-probiotic cell interaction assay

Micro titration plate containing biofilm of *Candida albicans* was taken. With the help of micro pipette, 50 µg of MRS broths was added up to the 10th well of each 3 row A, B and C. 50 µg of *Lactobacillus acidophilus* cell suspension was added in the first well of row A and made it 2 times concentration up to 10th well and after 10th well 50 µg cell suspension was discarded. 50 µg of *Lactobacillus plantarum* cell suspension was added in the first well of row B and made it 2 times concentration up to 10th well. 50µg of *Lactobacillus casei* cell suspension was added in the first well of row C and made it 2 times concentration up to 10th well.11th well was remained as positive control having only fungal biofilm of all the 3 rows. 12th well was remained as negative control having only normal saline of all of 3 rows. The titration plate was covered with lid. The plate was incubated for 48 hours at 37 °C. After 48 hours the OD values at 570 nm of each well were measured of all 3 rows (A, B and C). OD value of each well of each row was compared with the positive control well of each row to check the probiotic effect of *Lactobacillus* species.

3. RESULTS AND DISCUSSIONS

3.1 Isolation and identification of Lactobacillus species

In the current study, *Lactobacillus* species were isolated from different fruits and vegetables source collected from different shops located in Faisalabad. Isolated and identified different Lactobacillus species

were used to evaluate their probiotic potential against the *Candida albicans* which was isolated from the high sugar fruits and vegetables.

3.2 Macroscopic examination of Lactobacillus plantarum and Lactobacillus casei

Round and off white in color of colonies were appeared on the agar in Fig. 1(a). Small round, creamy white and rough surfaces colonies were appeared on the agar in Fig. 1(b).

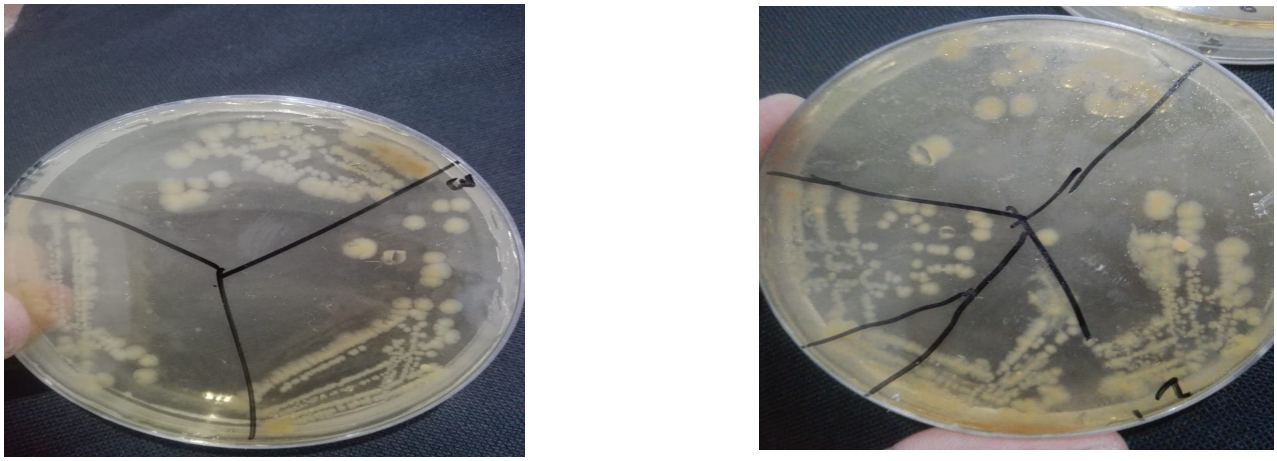

Fig.1. (a) Cultural features of *Lactobacillus plantarum* from orange. **Fig.1. (b)** Cultural features of *Lactobacillus casei* isolated from banana**.**

3.3 Microscopic examination of Lactobacillus plantarum and Lactobacillus casei

Microscopic examination revealed that *Lactobacillus plantarum* were Gram positive, rod and curved in shape. *Lactobacillus casei* displays as single and mostly in bunch and showed straight short rod.

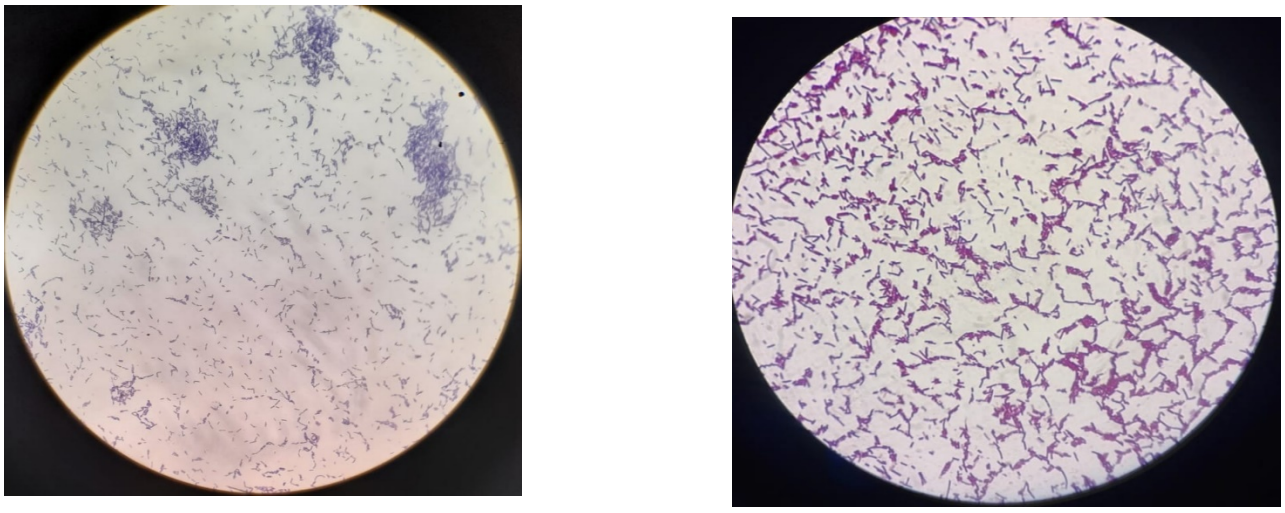

Fig. 2(a). Microscopic characteristics of *Lactobacillus plantarum* isolated from orange (40X) and in **Fig. 2(b)** Microscopic characteristics of *Lactobacillus casei* isolated from banana are shown.

3.4 Macroscopic and microscopic examination of Candida albicans

Creams to yellowish, smooth, round, and glistening colonies of *Candida albicans* were appeared on the *Sabouraud Dextrose Agar*. Under the microscope *Candida albicans* were appeared as abundant branched pseudohyphae and true hyphae with blastconidia are present.


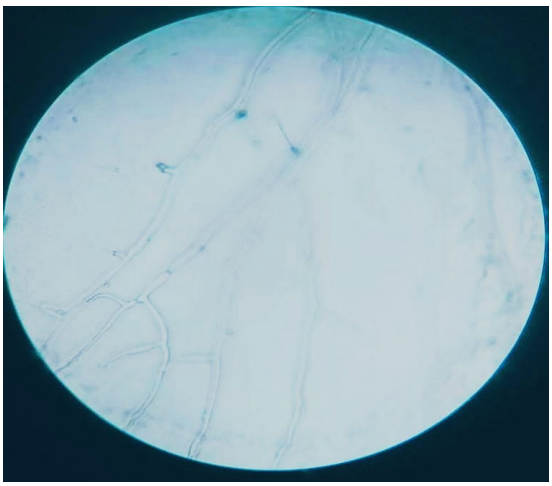

Fig. 3(a). Colony morphology of *Candida albicans* isolated from mango and in **Fig. 3(b).** microscopic characteristics of *Candida albicans* are shown.

4. Candida albicans biofilm production

Candida albicans' biofilm was observed after staining with lactophenol cotton blue in all the wells of titration plate except 12th which was as negative control.

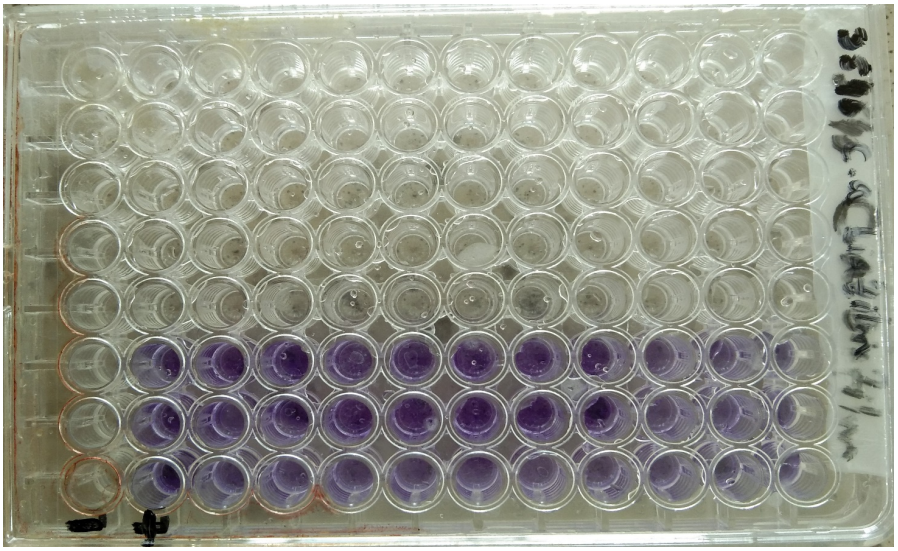

Fig. 4. Biofilm of Candida albicans

5. Probiotic assay

After measuring the OD value of all the wells of the microtitration plates at 570 nm *Lactobacillus casei* showed high anti biofilm activity followed by *Lactobacillus plantarum* and *Lactobacillus acidophilus* showed least anti-biofilm activity. After collection all the data, data was processed by two-way ANOVA statistical procedure.

Table 1. ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.1578	2	0.07889	F (2, 27) = 21.27	P<0.0001
Residual (within columns)	0.1001	27	0.003708		
Total	0.2579	29			

6. Probiotic Inhibition of Candida albicans species

Lactobacillus species induced a significant inhibition ($p<0.05$) in biofilm growths of *Candida albicans*. The antibiofilm potential of all three *Lactobacillus* species was significantly ($p<0.001$) different from each other with *Lactobacillus casie* inducing maximum biofilm inhibition.

Table 2. Comparison of standard means of Lactobacillus species

Groups	N	Mean ± SEM
Lactobacillus acidophilus	10	0.22 ± 0.03
Lactobacillus plantarum	10	0.10 ± 0.01
Lactobacillus casie	10	0.05 ± 0.01

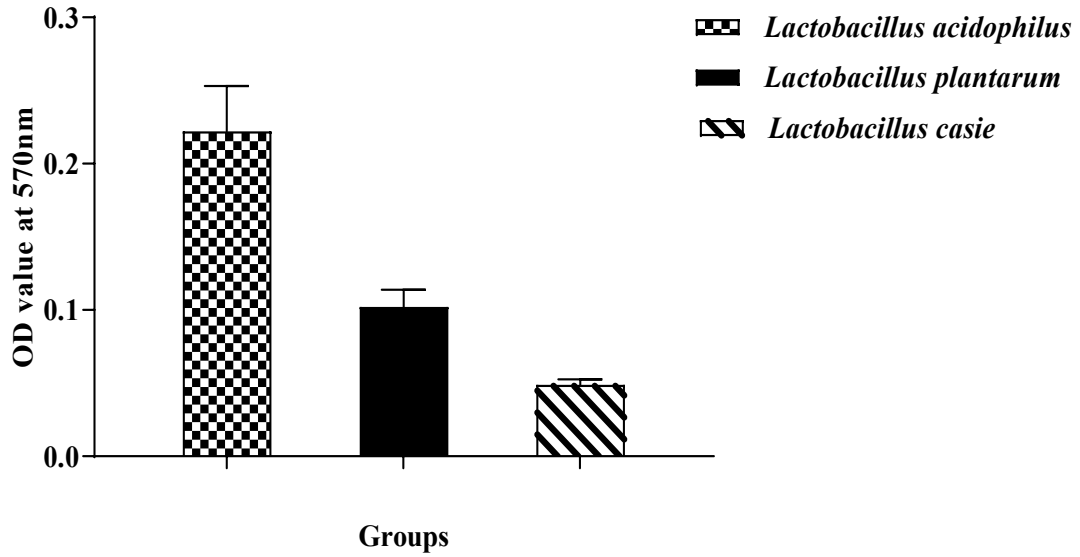

Fig. 5 Optical density of different bacteria

The resistance of pathogenic Candida species to common antifungal agents, rise in incidence of candidiasis in patients with compromised immune system and frequently repeated disease relapses demands some useful therapeutic approach. The use of probiotics for preventing, controlling and treating disease conditions is being suggested as an interesting alternative therapy[16]. The antimicrobial activity potential of lactic acid bacteria (LAB) species is well reported. The anticandidal activities and probiotic potential of many *Lactobacillus* species have been investigated[17]**.**

It is examined that Lactobacillus species induce inhibition of biofilm formation of *candida albicans*, an opportunistic fungal pathogen. The qualitative and quantitative analyses were performed using lactobacilli and biofilm of *Candid albicans* in a microtitration plate. *Lactobacillus plantarum, Lactobacillus casie* and *Lactobacillus acidophilus* isolated from local fruit and vegetable sources were analyzed in a probiotic assay for their ability to inhibit biofilm growths of *Candida albicans*.

By statistical analysis it was discovered that *Lactobacillus casie* induced maximum inhibition in biofilm growths of *candida albicans*. Biofilm growths of Candida albicans were sensitive to cultures of *Lactobacillus* species possessing antifungal activity spectrum. There was a significant difference among the means of inhibitions induced by three tested lactobacilli strains at different colony concentrations. It was concluded based on results analysis of this study that *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casie* exhibit anticandidal activities by inhibiting biofilm growth potential. The difference in biofilm inhibition potential of Lactobacillus species is likely due to specie and strain specific potential of producing antimicrobial substances[18].

The anticandidal activity of *Lactobacillus* species depends upon various overlapping mechanisms. The antibiofilm capacity of *Lactobacillus* against *Candida albicans* is based on competition for binding sites in host and production of organic acids, hydrogen peroxide and diacetyl bioactive compounds[19]. There are also studies that have reported the ability of *Lactobacillus* species to trigger immune response in hosts by interacting with immune cells production and releasing immune regulatory cytokines[20].

This study reveals that *Lactobacillus* species suppress the growth of *Candida albicans* biofilm most likely by curbing colonization and subsequent hypha formation due to action of exometabolites produced by lactobacilli. The direct contact of *Lactobacillus* cultures was essential for exhibiting antibiofilm effects against biofilm growths of *Candida albicans*. It is likely that yeast biofilm inhibition is caused by the acidic environment induced due to production of organic acids or due to production of bacteriocin-like substances[21].

There is still needed to explore possible reasons for marked capacity of *Lactobacillus casie* in comparison with other two tested strains of lactobacilli. The molecular mechanisms responsible for probiotic activity of lactobacilli against *C. albicans* are still undefined. The future investigations must be targeted for identification of efficacious components in exometabolite substances produced by lactobacilli exhibiting anti-candidal spectrum. The isolated components of exometabolite substances need to be assessed for their effects on expression of genes involved in biofilm filamentation.

4. CONCLUSIONS

The present study highlighted the probiotic potential of different species of Lactobacillus against the most pathogenic fungus Candida albicans. The Lactobacillus species had been identified by morphological, physiological, biochemical properties and similarly Candida albicans had been identified by morphological and microscopic characteristics. These isolates represented a potential source of species to be further studied in food sectors as preservatives concerning their antifungal activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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