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Evaluation of Antifungal Activity of Lactic Acid Bacteria against *Aspergillus flavus*

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Lactic acid bacteria (LAB) are considered as promising natural biological antagonists for mycotoxigenic fungi that contaminate various food commodities. A total 176 LAB isolated from different sources were screened against *A. flavus* by employing Dual culture assay method. Out of 176, two isolates *viz.*, LS-36 and LS- 44 showed highest inhibition of the toxicogenic fungi under *in vitro* conditions as well as reduction of fungal mycelium under *in vivo conditions*. Based on our previous studies, 16S rRNA based molecular characterization of these two isolates showed sequence homology of 99 to 100% towards *Lactobacillus brevis.*

Key words: Lactic Acid Bacteria, *Aspergillus flavus*, Antifungal activity, 16S rRNA.

A wide spectrum of filamentous mycotoxigenic fungi were identified as major food and feed spoilage agents worldwide[1]. Among them *Aspergillus, Fusarium, Penicillium* are more common mycotoxins producing fungal species[2]. *Aspergillus flavus* is recognized as one of the most dangerous fungi because of its ability to produce aflatoxin. Aflatoxin which is a group of polyketide-derived furanocoumarins[3] produced by a poliketide pathway and has toxigenic, mutagenic, teratogenic and carcinogenic properties and it's in toxication can result in death by suppressing the immune response[4]. Hence, occurrence of these molds in food is potentially dangerous for public health and cause major economic loss. In addition, they are also responsible for off-flavour, discolouration, rotting and disintegration of nutritional quality of the food which leads to sizable economic losses[5]. At present, physical and chemical methods are more popular to manage the occurrence of these molds and toxins produced by them, which are uneconomical and not efficient to reduce the presence of mycotoxins. So, identification of efficient, ecofriendly and cost effective biological agents inhibiting such dangerous molds in food is thus of primary importance. Bio-preservation, the control of one organism by another has received much attention from past few years[5].

Lactic acid bacteria (LAB) are a broad group of gram-positive, catalase-negative, non-sporulating and eco-friendly natural bio-antagonists recognized for several potential applications. From several studies, researchers corroborate that LAB has beneficial health effects in human beings. These bacteria have a long history of use in food preservation. As they produce some antagonistic compounds which control pathogenic fungi and also involved in detoxification of mycotoxin. Naturally, LABs are predominant in a diverse habitat like fruits,

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vegetables, milk, milk products and soil etc. and may provide great scope to isolate potential LAB strain(s) which was not studied earlier. But in the past, limited research has been done to identify potential LAB strains that efficiently control growth of mold and improve the shelf life of many fermented products and thereby reduce health risks due to exposure to mycotoxins[6]. With this background, the present study was designed to identify the potent lactic acid bacteria (LAB) from natural sources which are antagonist to *A. flavus*.

MATERIALS AND METHODS

Isolation and Selection of LAB

Lactic acid bacteria were isolated from various sources *viz.*, soil, fruits, grains, poultry Deep Litter, milk and milk products collected from local market and farmers field (Dharwad, Karnataka) by using Mann Rogosa Sharpe Agar (MRS) medium. The serial dilution plate count technique was employed in isolation of LAB. The dilution was carried out up to 10^{-6} dilutions. Aliquots (o.1 mL) of 10^{-4}, 10^{-5} and 10^{-6} were transferred to sterile petri-dishes and the molten and cold MRS was poured. The plates were incubated anaerobically in an inverted position for 3-5 days at 30^0C. Further, preliminary characterization was done based on colony morphology and microscopic observations using phase contrast microscope (Shimadzu, Japan).

Isolation of *Aspergillus flavus*

The grains of maize, cotton and groundnut infected with fungi were selected and placed on Potato Dextrose Agar (PDA) amended with streptomycin 100 mg/ml and incubated at 25^0C for two days to select the fungus *A. flavus*. The colonies which showed usually spreading and very light yellow to deep yellow green, olive brown were selected and further confirmed by microscopic observation using phase contrast microscope for fruiting body and spore characters. The fungus which has produced compact to radiate conidial heads with green shade were selected, purified and maintained on PDA and used for further analysis.

Screening of Lactic acid bacteria isolates against *Aspergillus flavus*

A total of 176 LAB isolates were isolated from different sources and tested for their antifungal activity against *A. flavus* in an initial screening step and dual culture assay was employed to detect the antifungal activity[7]. Freshly prepared spore suspension of *A. flavus* was inoculated into the plates containing Potato Dextrose Agar (PDA). Thereafter, discrete spots of 50 µl (containing about 105 bacterial counts per ml) of LAB isolates were placed on the PDA agar plate inoculated with *A. flavus* and kept for incubation at 30^0C for a period of 4-5 days. The plates were examined for the inhibition zone around the bacterial colonies and the zone of inhibition was measured.

In vivo analysis

Preparation of LAB inoculums

The Lactic Acid bacterial culture (LAB) was grown in 100 mL of MRS medium in 250 mL Erlenmeyer flask for 18 h at 37°C and used further for fungal inhibition analysis.

Preparation of spore suspension of *A. flavus*

Spore suspension was prepared from four days old cultures of *A. flavus* grown on PDA. The plates were flooded with 20 ml of sterilized doubled distilled water and brushed thoroughly for 1–2 min. under laminar air flow. The suspension was filtered through two layers of sterilized cheese cloth to remove the mycelia residues and collected in the sterilized flask and used for further experiments.

Fungal biomass inhibition analysis using potent LAB isolates

Based on the preliminary screening, the efficient LAB isolates were selected for fungal biomass inhibition analysis. One ml of freshly prepared LAB cultures were inoculated in 250 ml flask containing PDB and kept on the shaker at 28°C for 12 hrs. Thereafter, next day known quantity of four days old spore suspension of *A. flavus* was inoculated in the same flask and again kept on the shaker at 28°C. Untreated flask inoculated only with *A. flavus* was considered as a control. Observations were recorded at three days interval. The inhibition activity was determined by analyzing dry weight of the mycelia measured from each flask. Fungal growth was collected on Whatman No.1 filter paper (Whatman International, Maidstone, England) and dried in an oven at 50°C for 2 days. The average fungal biomass was calculated individually for each isolate treated with fungus and compared with the fungal biomass of uninoculated control.

Molecular characterization of the LAB isolates

The efficient *Lactobacillus* isolates LS-36 and LS-44 were analyzed for 16s rRNA gene sequence in earlier studies[8]

RESULTS AND DISCUSSION

Isolation and Phenotypic identification of LAB isolates and *Aspergillus flavus*

Overall, seventeen samples including fruits, seeds, poultry deep litter, milk and milk products were used for isolation of LAB. Among these samples, soil, poultry deep Litter, milk and milk products showed more number of isolates as it suggest predominant occurrence in these sources whereas, least number of isolates were recorded in fruits and seeds samples (Table 1), the colony morphology and microscopic characteristics of the isolate LS-36 and LS-44 illustrated in (Fig. 1). In general, it is said that LAB require complex nutritional requirements and therefore they are usually associated with nutrient-rich environments such as animal bodies, plants and foodstuffs etc[9]. In the past, several successful efforts have been done to isolate LAB from fruits, vegetables, milk and milk products. This is a new study which reports the use of poultry deep Litter as a source for assessing the predominance of LAB.

Table 1. Lactic acid bacteria (LAB) isolated from different sources

Sl. No.	Source	Location	No. of isolates
1	Khava	Local Market, Dharwad	12
2	Curd	Local Market, Dharwad	20
3	Buffallo Milk	Farmers House, Dharwad	8
4	Goat milk	Farmers House, Dharwad	10
5	Soil	MARS, UAS, Dharwad	54
6	Groundnut	Local Market, Dharwad	5
7	Apple	Local Market, Dharwad	6
8	Custard Apple	Local Market, Dharwad	2
9	Papaya	Local Market, Dharwad	1
10	Sporolac	Medical Store, Dharwad	1
11	Grapes	Local Market, Dharwad	21
12	Poultry Deep Litter	Poultry Farm, UAS, Dharwad	36
		Total no. of isolates	176

Table 2. Antifungal activity of LAB isolates against *A. flavus*

SI. No.	Isolate code	Inhibition zone (mm)
1	LK-5	15
2	LCU 3	16
3	LCU 18	16
4	LS 36	21
5	LG 4	17
6	LA 5	16
7	LS 44	22
8	LBM 4	17
9	LBM 7	17
10	LGO 6	17
11	LSP 6	17
12	LGR 9	16
13	LDL 4	18
14	LDL 12	19
15	LDL 13	18
16	LAB 75	19

Total of 359 bacterial colonies were selected from the plated MRS medium. Of them, 176 were characterized by selective enumeration for showing typical LAB characteristics. Primarily, all isolates were identified on the basis of colony morphology such as circular and cream in colour suggested relatedness of these isolates with lactic acid bacteria and further confirmed by gram staining test. Microscopically, all the cells were either rod or bacillus shaped arranged in pairs or tetrads. Similar results were also recorded by Kadere[9] and Hoque[10]. However, in the present study the cells of all the isolates showed deep pink colour upon gram staining which gave more evidence towards their confirmation as they are belongs to the lactic acid bacteria *species*. Saguibo[11] also observed circular, entire, opaque but shiny, cream-colored

colonies of lactic acid bacteria isolated from guava fruit.

In addition, isolation of *Aspergillus flavus* was done from fungus infected grains on PDA medium and it showed mycelium with compact radiate conidial heads in shades of dark green (Fig. 2). Similar observations were reported by Nazir[12]. Further, microscopic observations (Using Phase Contrast Microscope) of mycelium showed apically swollen conidiophores with numerous conidia-bearing cells (Fig. 3). Conidiophores were heavy walled, hyaline, coarsely roughened.

Screening of LAB isolates against *A. flavus*

All the 176 LAB isolates tested *in vitro* for antifungal activity against *A. flavus* revealed varying degrees of inhibition (ranged from 15 mm to 22 mm). Among them, 16 isolates were showed good inhibitory activity (>15mm) against the fungi (Table 2). Two isolates *viz*., LS 36 (21 mm) and LS 44 (22 mm) showed highest inhibitory action against the *A. flavus* (Fig. 4). This suggests that antifungal nature of LAB could be the results of many organic acids such as lactic and acetic acids and secondary metabolites produced by them. *Magnusson*[13] opined that an antifungal activity of LAB is influenced by several other components. The ability of individual isolates in producing the antifungal compounds is largely depending on the strain, cultural condition and growth media composition[14]. Singh[15]observed the variation in antifungal property of the same strain in different environments and stated that bacterial stress response rely on the coordinated expression of genes that alter different cellular processes like cell division, DNA metabolism, housekeeping, membrane composition, transport, etc. However, the results obtained in the present study were comparably higher than the results obtained in earlier studies[16]. The two isolates which showed highest inhibition against *A. flavus* were used for further *in vivo* analysis.

Fig.1.

Fungal biomass reduction (dry weight) analysis using identified potent LAB

Based on the *in vitro* growth suppression experiment *in vivo* analysis was undertaken. To

Fig. 2.

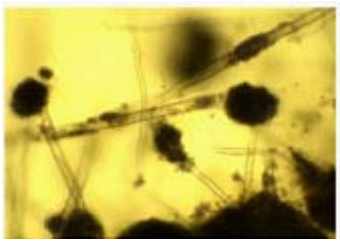

Fig. 3 .

Table 3. Effect of LAB on mycelial dry weight of *Aspergillus flavus*

Strain	Mycelial dry weight of *Aspergillus flavus* (in mg.)							
	6 days	% reduction . in wt	9 days	% reduction in wt.	12 days	% reduction in wt.	15 days	% reduction in wt.
LS 36	0.88	89.51	2.21	79.44	3.07	72.04	3.82	74.60
LS 44	0.35	95.82	0.56	94.79	0.92	91.62	2.19	85.43
Control	8.39		10.75		10.98		15.04	
C D@5%	0.38		0.18		0.30		0.17	
CV	0.82		2.96		4.47		1.70	
SE	0.01		0.06		0.10		0.06	

analyze the fungal biomass reduction, the two efficient LAB isolates viz., LS-3 and LS-44 were tested in Liquid MRS Medium and it showed significant reduction in mycelial growth compared to uninoculated control (Fig. 5). It can be concluded that when competitive organisms are present in medium, growth is suppressed. However, absence of non-competitive organisms in medium results in the normal or high growth. From the graph, it can be seen that the growth of *A. flavus* was suppressed from the beginning. Weight reduction (in grams) was observed very low in early stages and increased in later stages. In contrast, per cent weight reduction was observed high (89.51 and 95.82 for LS-36 and LS- 44, respectively) in earlier stage (6 days after inoculation) and later found decreasing(~74 to 72 for LS 36 and 85.43 for LS 44) in later stages (9, 12 and 15 days after inoculation) (Table 3). It is said that LAB evolves specific mechanisms to respond environmental stresses which help them to survive better when competition with other species. In general, when bacteria compete with fungi for nutrition in batch culture, bacteria succeed to grow faster over fungi as the metabolic activity of bacteria is fast as compared to fungi and also they are more simple living entity compared to fungi. This factor enable bacteria to use available nutritients earlier for growth which leads faster growth, while fungi develop later when nutrient become less available which caused poor growth compared to normal.

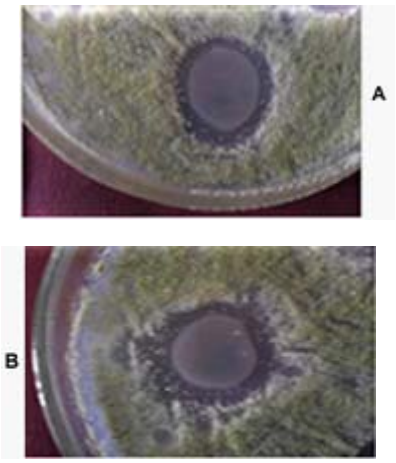

Fig. 4 .

Fig. 5 .

Moreover, many studies concluded, fungal inhibition by LAB also depended on the type of fermentative group to which they belong i.e., homo or hetero fermentative type and their end products such as organic acids (lactic and acetic acids) as well as carbon monoxide, respectively. Also, Gerez[17] explained, growth inhibition of fungus is directly proportional to increase in organic acid. Increase in organic acid may increase medium acidity which affected the normal metabolism and lead to fungal growth inhibition. The present findings are related with Aryantha and coworkers study [18], where they observed the presence of lactic acid in the medium becomes an inhibition factor for the growth of *A. flavus*. In addition to that, competition between lactic acid bacteria and fungi for available nutrients said to be important reason for growth inhibition of fungi.

Molecular identification of potent LABs isolates

The 16S rRNA based molecular characterization of both the potent isolates (LS-36 and LS-44) followed by Nucleotide BLAST analysis of sequenced product showed 99 % of homology towards *Lactobacillus brevis.* The 16S rRNA gene sequences were obtained from the promising LAB isolates LS-36 and LS-44 are submitted to NCBI Genbank under the accession numbers KM091440 and KM091441, respectively.

CONCLUSIONS

The two potential LAB strains LS-36 and LS-44 were identified which are effective in suppressing the growth of *A. flavus* a potential food and feed spoiling fungi. The molecular analysis of LS-36 and LS-44 indicates sequence homology of 99 % to *L. brevis*. These two strains will be further employed in further detoxifying the toxins produced by *A. flavus* in poultry feed.

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