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Assessment of microbial quality and antifungal property of native Lactic acid bacterial isolates of finger millet

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Abstract
Finger millet is the staple food product of low socio-economic sector in India. Even being listed in the major consumer product now It's storage and consequent spoilage of grains is an issue. Biological factors, mainly fungal pathogens play a major role for spoilage of finger millet products. In the present study Lactic acid bacterial isolates obtained from finger millet and *Lactobacillus plantarum* afp3, an antifungal strain of paddy were tested against common grain spoilage fungus, *Aspergillus niger* and the aflatoxigenic *Aspergillus flavus*. *Lactobacillus plantarum* afp3 showed highest inhibitory activity compared to the native isolates of finger millet in agar over lay method against both the test mold, *Aspergillus flavus* and *Aspergillus niger*.

Keywords: Antifungal, finger millet, lactic acid bacteria (LAB), *Lactobacillus plantarum*, *Aspergillus flavus*, *Aspergillus niger*

1. Introduction
Millets are the coarse grains that are rich in fiber, proteins, vitamins and minerals (Ramashia *et al.*, 2019) [16]. Among which finger millet (*Eleusine coracana*) also known as '*ragi*' is gluten free millet commonly consumed in South India. The grains contain a high amount of minerals like calcium, phosphorus and zinc (Gebre *et al.*, 2019). This is a protein diet for infants, elderly as well as for nutritionally deprived people. India is known for different finger millet-based food products like *roti*, *dosa*, porridge, muddle and *ambali* or *koozh* (Aliya and Geervani, 1981; Vaidehi *et al.*, 1985) [1, 19]. Although, the food products of finer millet reached the commercial market reveal the presence of various fungal contaminants and related mycotoxins in processed flour. Mycotoxins like fuminosin (Penugonda, 2011) [18], ochratoxins (Kibebe, 2018) [11] and aflatoxin (Senthilkumar *et al.*, 2021) [17] were reported in millet flour, and which constitutes a greater threat to the human health.
The need for biopreservation of processed foods has gained its popularity. There are different methods for preservation and production of toxin free millet products. As the preference for naturally preserved food products is growing by consumers antifungal Lactic acid bacteria (LAB) as a method of biopreservation in food products have been the subject of numerous studies over the years (Dopazo *et al.*, 2021; Khalil *et al.*, 2021) [3, 10]. Antifungal LAB as a protective culture in various food products likes bread (Jin *et al.*, 2021) [9], cheese (Makki *et al.*, 2021) [13] and yogurt (Xu *et al.*, 2021) [21] is reported. In the present study evaluation of the antifungal activity of native LAB isolates of finger millet grains in comparison with antifungal strain of paddy *Lactobacillus plantarum* afp3 under *in vitro* condition was carried out to use on finger millet preservation and the results are reported.

2. Materials and Methods
2.1. Sample collection
Finger millet grains (var CO 14) stored for about one month after harvesting were collected from the farm house at the Tamil Nadu Agricultural University in Coimbatore, India. The millet grains were stored in polyethylene bags for further studies.

2.2. Determination of microbial quality of finger millet grains
Ten gram of finger millet grain was mixed with 90 ml of sterile water to give 10^{-1} dilution and serially diluted further. Appropriate dilutions were plated on Man, Rogosa and Sharpe (MRS) agar, Tryptone soy agar (TSA) and Potato dextrose agar for Lactic acid bacteria, aerobic

bacteria and fungi respectively (Harrigan, 1976) [7]. Plates were incubated aerobically at 30 ± 2˚ C for 24-48 h for LAB, at 24 ±2˚ C for 4-5 days for fungi and at 32 ±2 ˚ C for 24 h for aerobic bacteria. Colonies were observed for variations in morphology and enumeration was done.

The LAB isolates were purified by repeated streaking on MRS agar plates. A series of morphological tests were performed on the Lactic acid bacterial isolates. Gram staining was used to determine the Gram reaction of LAB isolates. Under a microscope, the cellular morphology, such as shape and cell arrangement, was studied. In MRS agar media, colony characteristics such as color, elevation, and shape were observed and data were reported (Gerhardt *et al.*, 1981) [6]. Fungal isolates were purified by single spore isolation method and was identified by colony morphology and microscopic observation (Gaddeyya *et al.*, 2012) [5].

2.3. Cultures and growth condition

An antifungal isolate of *Lactobacillus plantarum* afp3 originally isolated from paddy, and LAB isolates obtained from finger millet grains were used for the antifungal activity study. LAB isolates were stored at -80˚C in MRS supplemented with 20%glycerol and routinely cultured in MRS broth and incubated aerobically at 37 ± 2 ˚C for 24 to 48 h (Pérez-Cataluña *et al.*, 2018) [15]. *Aspergillus flavus* and *A. niger* were obtained from culture bank of the department and routinely cultured on PDA medium at 24 ± 2˚C.

2.4. *In vitro* antifungal activity test for *L. plantarum* afp3 andnative LAB isolates

In vitro test for antifungal activity of *L. plantarum* afp3andnative LAB isolates of finger millet grainsagainst *A. flavus* and *A. niger* was carried out by agar overlay method (Fernandez *et al.*, 2017) [4]. 5 µl of actively growing LAB cultures on MRS broth was spotted on MRS agar plates. The culture plates were incubated for 2 days at 30˚C aerobically (Meroth *et al.*, 2003) [14]. After 2 days incubation plates were overlaid separately with 10 ml of YEG medium containing 1% agar with 10^4 fungalspores/plate. The plates were then incubated for 7-14 days and observed for mould growth. The antifungal activity was measured based on the diameter of inhibition zone around the LAB colonies.

2.5. Compatibility test between *L. plantarum* afp3 and native LAB isolates

The compatibility between *L. plantarum* afp3 and native LAB isolates were done by streak line procedure on MRS agar plate by using method given by Annuk *et al.* (2003) [2]. A single line of *L. plantarum* afp3 was streaked on the MRS agar plates. The native LAB isolates of finger millet grains were grown on MRS broth and seeded perpendicular to the streak line of *L. plantarum* afp3. The plates were incubated aerobically at 37 ± 2 ^{0}C for 24 to 48 h and observed for development of any inhibition zone at the point of intersection of two isolates.

3. Results and Discussion

3.1. Microbial quality of finger millet grains

The microbial quality of the finger millet was determined by serial dilution and plating technique. An aerobic bacterial population of 5.87±0.01 log cfu mL^{-1} and 3.04±0.04 log cfu mL^{-1} of fungi were found to be present in the sample. LAB population was low with about 1.38±0.01 log cfu mL^{-1} in the sample. The fungal isolates obtained from the sample were identified by colony morphology on agar plates and also microscopically.*A. flavus* was found to be in higher population (33%) than *A. niger* (31%)and *Fusarium oxysporum* (22%) on the agar plates when calculated out of total number of fungal colonies (Figure 1).

Aspergillus, *Fusarium*, and other fungal pathogens can affect grains both before and after harvest. *Aspergillus* is a xerophilic fungus with capacity to grow under low water activity (a_w below 0.6). Water activity will be minimal during storage, and *Fusarium* will not be able to produce mycotoxins at that point; nevertheless, *Aspergillus*, a frequent storage fungus, is known to produce mycotoxins (Hocking, 2003) [8]. The source of xerophilic mould contamination is storage sheds themselves (Wicklow, 1995) [20]. The sample used in this study was stored grains; and the greater *Aspergillus* population was attributable to contamination that occurred during storage. LAB population was very low in the sample used in this study. But different morphological forms appeared on MRS agar plates indicating heterogeneity of lactic acid bacterial members By repeated streaking for three times on MRS agar plates 20 pure culture of LAB were obtained. Based on the colony morphology, Gram's reaction, catalase test and shape of the cell, the LAB isolates were classified into five groups and the results were given in table 1. All the isolates were gram positive in nature and showed negative response to catalase test. Among the isolates 60% were found to be cocci, and remaining 40% belongs to bacilli form.

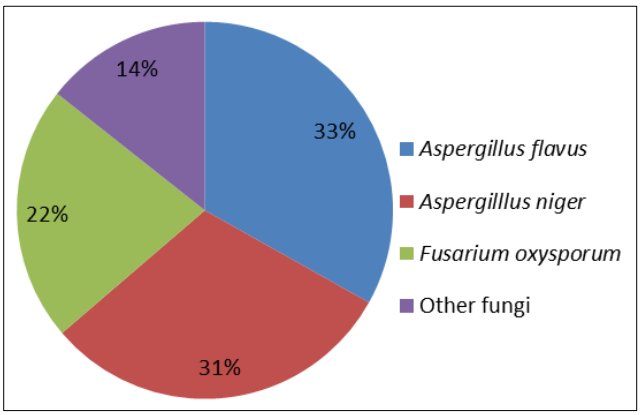

Fig 1: Occurrence of different fungal genus on finger millet grains (var. CO 14)

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3.2. Antifungal activity of native isolates from finger millet and *L. plantarum* afp3

Agar over lay method was employed to assess the antifungal activity of native population of LAB in finger millet, against the two mold species *viz.*,*A. flavus* and *A. niger*. Antifungal strain *L. plantarum* afp3 of paddywas compared with the native isolates for the antifungal potential. The native isolates showed very low inhibitory activity against *A. flavus* and *A. niger*, but *L. plantarum* afp3 exhibited higher level of inhibition (Table 2). The inhibition zone diameter for *L. plantarum* against *A. flavus* was found to be about 3.22 ± 0.19

cm against *A. flavus* and 1.77 ± 2.7 cm against *A. niger* (Figure 2). Leyva Salas *et al.* (2018) also screened different antifungal LAB isolates previously available for use as bio protective culture in yogurt and cheese. Here in this study since the native isolates is having nil/ very low antifungal activity *L. plantarum* afp3 with good antifungal activity can be used for preservation of millet flour.

Table 1: Colony morphology, microscopic and biochemical character of LAB isolates obtained from finger millet (var. CO 14) grains

S. No.	Colony morphology				Microscopic characters		Catalase test	Isolate No.
	Color	Elevation and edge	Size	Form	Shape of cell	Gram's reaction		
1.	Pale white	Convex, entire	+	Circular	Cocci	Positive	Negative	LABR12, LABR13,LABR18
2.	Creamy	Convex,irregular	++	Irregular	Cocci	Positive	Negative	LABR4, LABR6, LABR3, LABR11, LABR16, LABR15, LABR20
3.	White	Flat,irregular	++	Irregular	Streptobacilli	Positive	Negative	LABR1, LABR2, LABR9, LABR10, LABR14, LABR19
4.	Creamy white	Flat,irregular	+	Irregular	Cocci	Positive	Negative	LABR17
5.	Creamy white	Convex, entire	+	Regular	Diplobacilli	Positive	Negative	LABR5, LABR7, LABR8

Size of colony	
+++	Large
++	Small
+	Pin pointed

Table 2: Antifungal activity of native LAB isolates and *L. plantarum* afp3against *A. flavus* and *A. niger* by agar over lay method

Isolate No.	Test mould	
	A. flavus	***A. niger***
LABR1	+	-
LABR2	+	-
LABR3	-	-
LABR4	-	+
LABR5	+	-
LABR6	+	-
LABR7	-	-
LABR8	-	+
LABR9	+	-
LABR10	-	-
LABR11	-	+
LABR12	-	-
LABR13	-	+
LABR14	-	-
LABR15	+	-
LABR16	-	-
LABR17	-	+
LABR18	-	-
LABR19	+	-
LABR20	-	-
L. plantarum afp3	+++	++

+++: >30mm inhibition zone diameter; ++: 10-30 mm inhibition zone diameter; +: <10mm inhibition zone diameter; -: No inhibition zone

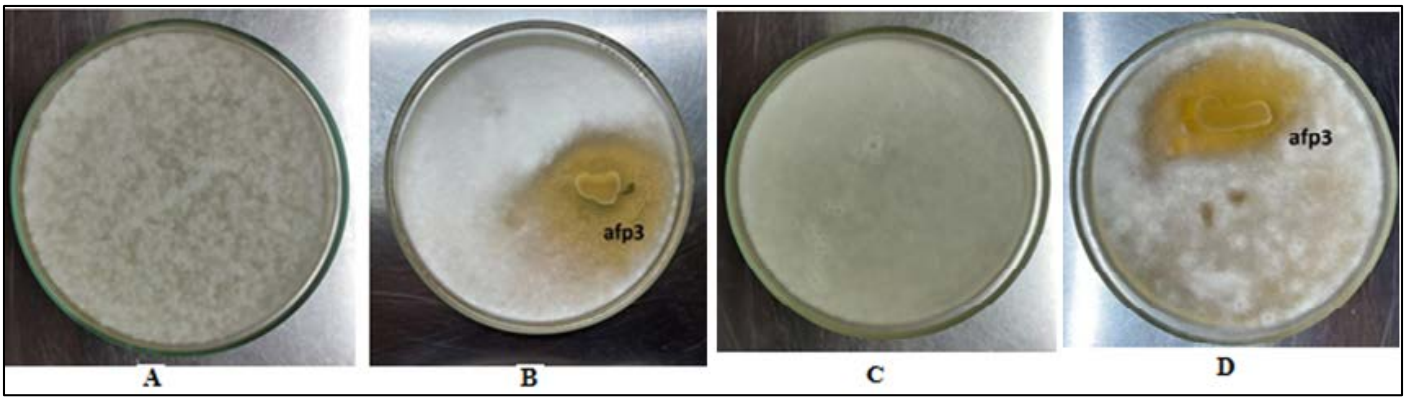

Fig 2: Antifungal activity of *L. plantarum* afp3 on MRS agar plate by agar over lay method against *A. flavus* and *A. niger*, A. Growth of *A. flavus* on MRS agar plate, B. *L. plantarum* afp3along with *A. flavus* after 14 days incubation, C. Growth of *A. niger* and D.*L. plantarum* afp3along with *A. niger*.after14 days incubation

3.3. Compatibility between native LAB isolates from finger millet and*L. plantarum* afp3

The antifungal isolate, *L. plantarum* afp3 to be used in finger millet flour for bio preservation, it must be compatible with the native LAB population in order to keep its natural quality. Hence the compatibility between the antifungal LAB culture *L. plantarum* afp3and native isolates was checked by cross streak method and the results were given in Figure 3. No inhibition zone was observed in the intersection of any two cultures. All the native isolates exhibited compatibility with *L. plantarum* afp3. The colony morphology of the LAB isolates when grown individually on MRS agar plates were compared with the cross streak assay plates and all the isolates showed similar colony morphology and growth. There is no inhibition activity in between native isolates and *L. plantarum* afp3.

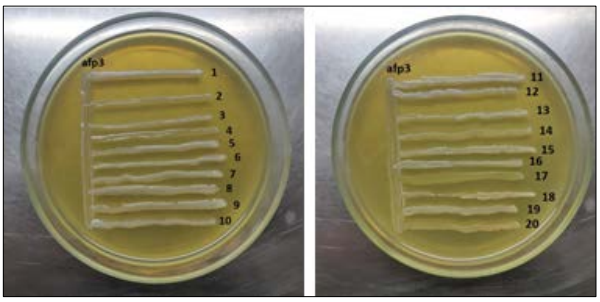

Fig 3: Compatibility test between *L. plantarum* afp3and native isolates designated LABR1 to LABR20 (1-20) from finger millet grains

4. Conclusion

Food spoilage by different molds is the principle cause of food deterioration and wastage. In the wake of increased demand for minimally processed natural food, chemical method of fungal control is kept aside and a natural solution offered by LAB and its metabolites is exploited widely. This study focused on the development of an antifungal culture for the preservation of finger millet flour against fungal spoilage. *Lactobacillus plantarum* afp3 strain identified for antifungal activity against two test molds *A. flavus* and *A. niger* in this study is compatible with the native LAB isolates in finger millet. So it can be used for the preservation of millet flour. The probiotic potential of this antifungal strain is to be established to realize its complete benefit.

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