RESEARCH ARTICLE

Preliminary studies on the *in situ* prevention of food spoilage fungi using antifungal Lactic acid bacteria

Preliminary study on the in situ prevention of food spoilage fungi using antifungal Lactic acid bacteria
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ABSTRACT

Food loss is a major threat in the world and one-third of the food produced for human consumption are wasted. Many fungal species cause loss of food/raw materials like grains, fruits and vegetables throughout the world. Chemical preservatives and fungicides when used in food have negative impacts on health and the environment. Biopreservatives such as lactic acid bacteria (LAB) are effective, safe, biodegradable and have additional health benefits. The focus of this research is to survey the occurrence of native lactic acid bacteria with antifungal activity in various food sources and to obtain a potential lactic acid bacterial isolate for strategic application to control fungal pathogen in food products. In the present study, the population of lactic acid bacteria were about 10^4 to 10^5 cells in all food samples. Sixty isolates of lactic acid bacteria were obtained from samples collected. Of these, 21 exhibited inhibition towards the growth of *Fusarium oxysporum* and 13 isolates towards *A. flavus*. Ten of the total isolates exhibited inhibition towards both the test fungi. Based on potential of antifungal activity in the dual plate technique, six isolates were selected and subjected to fungal growth inhibition analysis using microplate. Of these six isolates, the isolate LABT3 showed the highest inhibition percentage (%) against both the target fungi. The isolate was tested for its growth and maximum growth was noted at 48 h and pH at this period was 5.16.

Keywords: Lactic acid bacteria; *A. flavus*; *F. oxysporum*; Fungal growth inhibition; Food spoilage

INTRODUCTION

Postharvest loss refers to significant food loss in the postharvest system (Lucia, 1994). Life style change had made the food industry flourish and processed foods are ruling the food market. Raw agricultural products and processed food products like fruit juices carry many spoilage bacteria and fungi. In India, the production of raw food materials is about 450 million tonnes of which 10 % postharvest loss is in cereals and pulses, 20 % in semi perishable and 25 % in products like milk, meat, fish and egg (Rajasri *et al.*, 2014).

Fungi are known as one of the important spoilage organisms in food (Pitt and Hocking, 2009). The Food and Agricultural Organization reported that 25 % of worldwide crops were contaminated by mycotoxin producing filamentous fungi like *Fusarium*, *Aspergillus*, *Penicillium* (Joint *et al.*, 2002). To eliminate these mycotoxins, postharvest detoxification strategies like physical, chemical and biological strategies have been reported (Jard *et al.*, 2011).

The excessive use of fungal detoxicants leads to the development of a resistant pathogen population and a consequent increase in toxic residue in food products. Also, consumer preference for natural antimicrobial compounds of plant and animal origin has led to the search for bio preservatives to prevent or control fungal spoilage in raw and processed foods (de Souza Sant'Ana *et al.*, 2008; Walter *et al.*, 2015).

Lactic acid bacteria (LAB) have attained Generally Recognized As Safe status (Stiles, 1996) for food application. Antifungal metabolites produced by members of lactic acid bacteria are lactic acid, acetic acid, formic acid, propionic acid, fatty acid, hydrogen peroxide, diacetyl, cyclic dipeptides, reuterin and phenolic compounds have been documented (Crowley *et al.*, 2013). The cell-free supernatant of *Lactobacillus* sp. from the buttermilk of raw buffalo milk had strong and broad antimicrobial activity against both harmful bacteria and toxigenic fungi such as *Aspergillus parasiticus*, *A. flavus* and *A. carbonariuis* and also inhibited aflatoxin and ochratoxin production (Shehata *et al.*, 2019). The LAB isolated from fermented beverages were identified as *L. plantarum*, *L. paracasei* and *L. pentosus* showed strong antifungal activity against *Colletotrichum gloeosporioides* (Barrios-

Roblero *et al.*, 2019). Preservation of tomatoes with the cell-free supernatant of *L. plantarum* reduced the fungal spore load (Luz *et al.*, 2020). In the present study, LAB isolates with potential antifungal properties suitable for *in situ* application in food were screened.

MATERIAL AND METHODS

Collection of sample

The samples of curd and idly batter, fermented in the household for 12 h, fresh tomato and cabbage collected from Uzhavar sandhai and paddy grains collected from Central Farm, Tamil Nadu Agricultural University, Coimbatore were used. The samples were transferred to the laboratory in sterile plastic bags. Samples were not stored.

Fungal cultures

The fungal cultures of *Aspergillus flavus* AS4 (MT830910) and *Fusarium oxysporum* FOS CB1 (MN999964) were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The fungal cultures were sub-cultured on Potato Dextrose Agar (PDA) media by disc method. Fungi were incubated at 25±2 ºC for 4-5 days.

Preparation of samples

Approximately 5 g of paddy grains were soaked in 25 mL sterile water and incubated for 12 h at 28 ºC. The cabbage was cut into 1-2 cm pieces and 180 g was taken in a sterile airtight glass container and added with 2 % (w/v) salt, mixed and pressed tightly without the air space and incubated at 28 ºC for 5 days (Lee *et al.*, 2006). Tomatoes were cut into pieces and homogenized in the stomacher (Bag Mixer ®, Interscience, France) for 2 min (Elmabrok *et al.*, 2013).

Isolation of LAB from the prepared samples

For the isolation of LAB, 1 mL of curd, 1 g of idly batter, 1 mL of homogenized tomato sample, 1 mL of soaked water of paddy grains and 1 mL of fermented liquid of cabbage were taken. Appropriate dilutions were plated in MRS (de Man Rogosa Sharpe) agar plates (De Man *et al.*, 1960) by pour plate technique and the plates were incubated at 37 ºC for 48 h. After incubation, the various morphological colonies were selected and purified. All the isolates were tested for Gram reaction and catalase test.

Screening of LAB isolates for antifungal activity

LAB isolates were screened for antifungal activity by dual plate technique (Gerbaldo *et al.*, 2012). The isolates were seeded until covering one-third of the surface of MRS agar plates and incubated at 37 ºC for 48 h. A PDA plug with *A. flavus* and *F. oxysporum* was placed on the center of the free surface of the above LAB seeded MRS agar plates. The plates were incubated aerobically at 25±2 ºC for 5 days. The mold growth was observed daily and inhibition distance was recorded.

Microplate fungal growth inhibition analysis of LAB isolates

Preparation of fungal spore suspension

The fungal spores were cultured in PDA plates. The fungal spores were collected by flooding 5 mL of sterile distilled water on fully grown pure *A. flavus* and *F. oxysporum* plates. The sterile water with spores were collected as spore suspension. An appropriate dilution was made by serial dilution technique. Then the spores of both fungal culture was counted in hemocytometer and recorded as $4X10^5$ spores mL^{-1} for *A. flavus* and $3X10^5$ spores mL^{-1} for *F. oxysporum*.

Microplate fungal growth inhibition analysis

One percent inoculum containing 10^7 cells mL^{-1} of 24 h old culture of 6 LAB isolates was inoculated in MRS broth, incubated at 37 ˚C in shaken condition. After 48 h of incubation, bacterial biomass was separated by centrifugation at 8000 rpm for 15 min. The 10 µl of spore suspension with 160 µl of cell-free supernatant (CFS) of LAB isolates were taken in sterile microtiter plate; 10 µl of spore suspension with 160 µl of sterile MRS broth was maintained as control. The microtiter plate was incubated at 25±2 ˚C for 72 h. The antifungal activity of LAB isolates was evaluated by reading absorbance at OD_{490nm} for *A. flavus* and OD_{530nm} for *F. oxysporum* using a microplate reader (Synergy HTX). The results were represented in percentage and calculated by using the following formula (Rugirello *et al.*, 2019),

$$\text{Percentage of fungal growth inhibition} = \{(\text{Control} - \text{Treatment}) / \text{Control}\} \times 100$$

For statistical analysis, ANOVA followed by Duncan test was performed by using SPSS assessed using $p < 0.05$. The best performing isolate was identified by analyzing within the isolates for each target fungi and being significant from each other.

Growth characteristics of antifungal LAB isolate

The LAB isolate LABT3 was inoculated in 100 mL sterile MRS broth with initial pH of 6.4 and incubated at 37 ºC for 24 h. The 1 % (10^6 cells mL^{-1}) inoculum was used for the studies. The growth of cells was expressed in log cfu mL^{-1}. The viable cell population of the culture at different time intervals was recorded by plating the appropriate dilutions at periodical intervals on MRS agar plates. The cell-free supernatant was collected by centrifugation at 8,000 rpm for 15 min and pH of the culture broth was recorded by using pH meter (µ pH System 361, Systronics, India).

RESULTS AND DISCUSSION

Occurrence of LAB in agricultural products

Lactic acid bacteria (LAB) are ubiquitously present in most food systems. The presence of LAB in raw and fermented agricultural products and their role in bio preservation were documented (Schnurer and Magnusson, 2005). In our study, the samples of soaked water of stored paddy grains, idly batter, tomato, fermented cabbage and curd were found to carry a good population of LAB. In general, the fermentation process has increased the population of LAB. The population of lactic acid bacteria was high in fermented food samples such as curd ($83.5X10^5$), idly batter ($75.2X10^5$), fermented cabbage ($62.5X10^5$) than nonfermented food samples such as fresh tomato ($38.6X10^4$) and paddy grains ($28.3X10^4$) (Table 1). The colony morphology of the LAB isolates from the samples varied to a great extent in terms of colour from white, pale white, creamy and pale yellow, different forms of elevation and edges as convex, flat, raised and entire. A total 60 isolates were taken on the basis of colony morphology. All the isolates were positive to Gram staining reaction and catalase-negative. Of these 60 isolates, 51.66 % were cocci and 48.33 % were bacilli. The majority of the LAB isolates obtained from traditional butter were bacilli (52.34 %) compared to cocci (47.66 %) (Bettache *et al.*, 2012)., However, LAB isolates obtained from fermented rice were found to be cocci and diplococci than bacilli (Jeygowri *et al.*, 2015). The production of exopolysaccharide was observed in most LAB isolates obtained from soaked water of paddy grains.

Antifungal activity of LAB isolates

Plethora of papers documented the use of LAB as antifungal agents for fungal growth inhibition and detoxification of mycotoxins (Gourama and Bullerman, 1955; Muhialdin *et al.*, 2020). The sixty LAB isolates obtained in this study were screened for antifungal activity by dual plate technique. Among the 60 isolates, 21 isolates exhibited inhibition of growth of *F. oxysporum* and 13 isolates inhibited *A. flavus*. Both the fungi were inhibited by 10 of the total 60 isolates (Table 2). The fungal inhibition by LAB isolates was presented in Figure 1. The isolates of paddy grains showed a similar level of inhibitory activity against both the test fungi (Figure 2). Guo *et al.*, 2012 informed that, 77 % of LAB isolates obtained from cheese and sourdough inhibited at least one fungus and 43 % inhibited both *A. niger* and *A. fumigatus*. Our results found a higher percentage occurrence of antifungal LAB isolates in curd and idly batter than paddy grains and tomato. Based on the level of inhibition against both the test fungi, of the 24 isolates six isolates *viz.*, LABT3, LABC2, LABC7, LABP1, LABP2 and LABP3 were selected.

The fungal growth inhibition analysis was carried out for these six antifungal isolates using microplate reader. The isolate LABT3 showed the highest inhibition percentage of about 90.59 % against *F. oxysporum* and 86.69 % against *A. flavus* and was significantly different from other isolates in its inhibition level to fungi (Table 3). The LAB strains isolated from cocoa fermentation were analyzed by microplate reader for their antifungal activity against *Penicillium citrinum* M6E1TS, *P. griseofulvum* M2BT2, *P. griseofulvum* S2TC, *Aspergillus niger* DsfAn, *A. fumigatus* DsfAf and *A. flavus* ST2A and found that *Lactobacillus plantarum* LARB65 showed the highest inhibition percentage ranging from 88 to 100 % against all six target fungi (Ruggirello *et al.*, 2019).

Growth characteristics of the isolate LABT3

The viable cell population of the isolate LABT3 was determined by growing in MRS broth with initial pH of 6.4 at different time intervals. The isolate attained a maximum viable population of about 11.72±0.98 log cfu mL^{-1} at 48 h. At 48 h the pH was recorded as 5.16±0.11. The pH of the culture broth was further reduced to 4.09±0.09 at 72 h (Figure 3) and a reduction in cell number was noted at this time. *Lactobacillus plantarum* CCDM 181 attained a viable population of 9.46 log cfu mL^{-1} at 18 h and the pH of the culture broth was reduced earlier at 18 h to 4.01 (Horackova *et al.*, 2018).

Abbreviations

CFS – cell-free supernatant

cfu – Colony forming units

LAB—Lactic acid bacteria

CONCLUSION

The present study was conducted to select naturally occurring lactic acid bacteria with antifungal activity from different food systems. The LAB isolates with antifungal activity was high in fermented foods than in non-fermented foods. Under *in vitro* conditions the isolate LABT3 was identified as a potential isolate to inhibit the growth of *A. flavus* and *F. oxysporum*. Since these two fungi produce mycotoxins, the antifungal LAB isolate LABT3 obtained in the present study would be a potent LAB candidate for *in situ* application in the food system to inhibit fungal growth and detoxification.

Ethics statement

No specific permits were required for the described studies because no human or animal subjects were involved in this research.

Originality and plagiarism

We ensure that we have written and submitted only entirely original works, and if we have used the work and/or words of others, this has been appropriately cited.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflict of interest in the publication of this content

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required. If anything is required from the MS, certainly, this will be extended by communicating with the corresponding author through corresponding official mail; vijiladauphin@yahoo.co.in

Author contributions

Idea conceptualization and guidance- - KV; Experiments- CS; Writing-original draft- CS; Writing- reviewing &editing - KV, CS

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Tables

Table 1 Population of LAB in different samples

S.No.	Place of collection	Name of the sample	Description of the sample	Population of LAB (cfu mL^{-1} or cfu g^{-1})	No. of Selected isolates
1.	Central farm, Tamil Nadu Agricultural University, Coimbatore	Paddy grains (ADT 43)	Stored grains (2 months) soaked for 12 h	$28.3X10^4$	5
2.	Home made	Idly batter	Rice and black gram ratio is 4:1 and fermented for 18 h	$75.2X10^5$	15
3.	Uzhavar sandhai	Tomato	Fresh vegetable	$38.6X10^4$	10
4.	Uzhavar sandhai	Cabbage	Fermented for 5 days	$62.5X10^5$	10
5.	Home made	Curd	Fermented for 12 h	$83.5X10^5$	20

Table 2 Antifungal activity of LAB isolates against fungal contaminants by dual plate technique

S.No.	Isolate No.	*Aspergillus flavus*	*Fusarium oxysporum*
1.	LABT1	-	++
2.	LABT3	++	+++
3.	LABT4	+	+
4.	LABT5	-	++
5.	LABT8	-	+
6..	LABP1	++	++
7.	LABP2	++	++
8.	LABP3	+	++
9.	LABP4	+	+
10.	LABB2	-	+
11.	LABB3	++	-
12.	LABB5	-	++
13.	LABB8	+	-
14.	LABB9	-	+
15.	LABC1	-	+
16.	LABC2	+	+
17.	LABC3	-	++
18.	LABC4	+	+
19.	LABC6	+	+
20.	LABC7	++	+
21.	LABC8	-	+
22.	LABC10	-	+
23.	LABC11	-	++
24.	LABC15	++	-

Note

+++ : >2.5 cm inhibition distance

++ : 1 to 2.5 cm inhibition distance

+ : <1 cm inhibition distance

- : No inhibition

Table 3. Fungal growth inhibition by LAB isolates

LAB isolates	Fungal growth inhibition (%)	
	A. Flavus	*F. oxysporum*
LABC2	66.20±0.20[b]	75.90±0.09[b]
LABC7	65.50±0.68[b]	42.08±0.40[d]
LABP1	62.53±0.69[b]	40.70±0.82[d]
LABP2	63.53±0.69[b]	78.22±0.77[b]
LABP3	40.78±0.79[c]	71.25±0.13[c]
LABT3	86.69±0.97[a]	90.59±0.11[a]

Data are mean ± standard deviation; a/b/c/d referred to various data within isolates of each fungus

Figures and Structures

Figure 1: Fungal inhibitory activity of LAB isolates against *Aspergillus flavus* and *Fusarium oxysporum* (5 days of incubation)

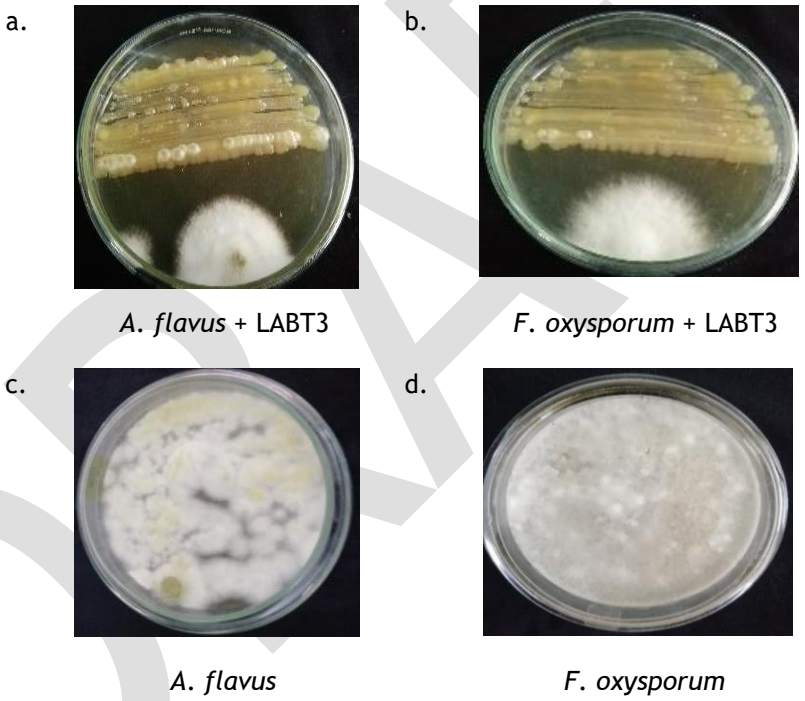
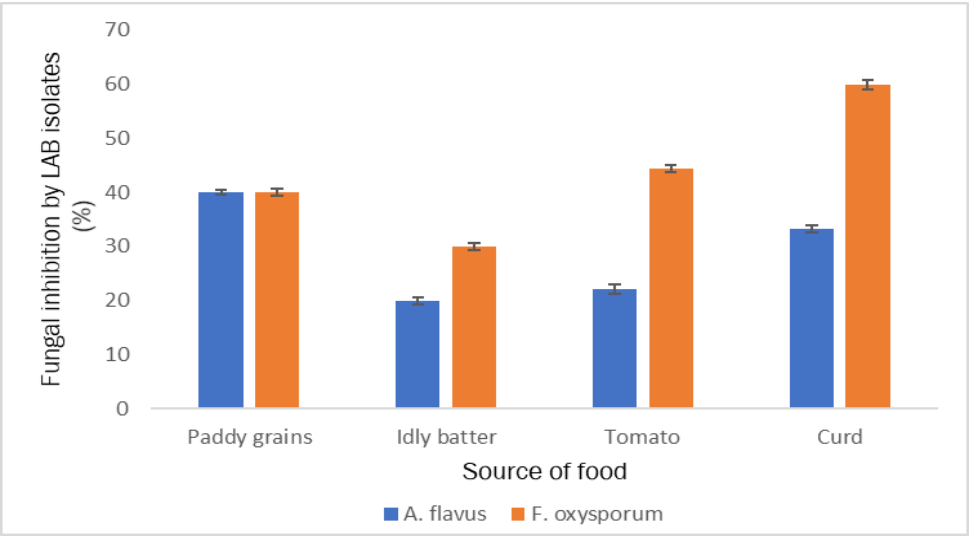
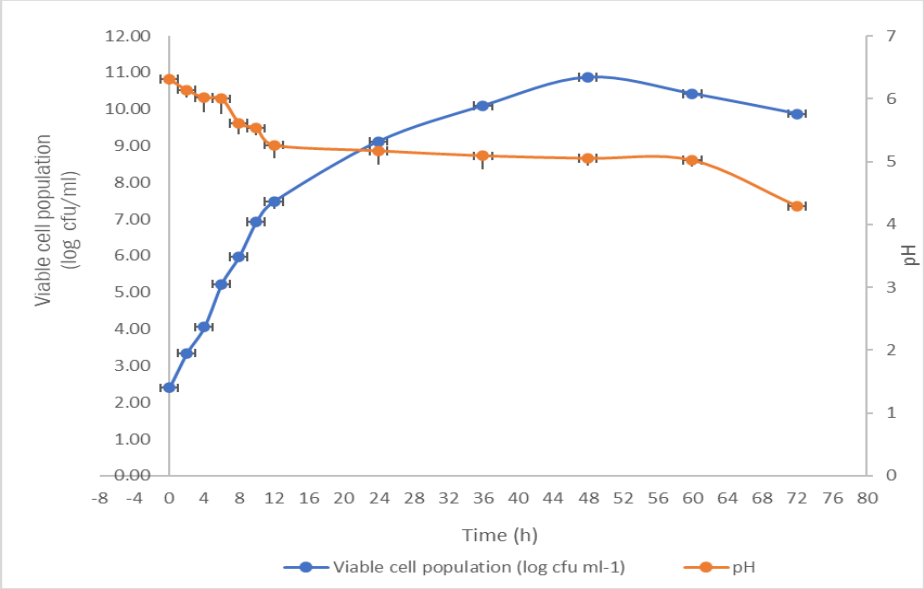


Figure 2: Percentage of fungal inhibition by LAB isolates from different samples



Error bars represent the standard error

Figure 3: Viable cell population of the isolate, LABT3 isolate at different time period



Error bars represent the standard error