Probiotics & Antimicro. Prot. (2019) 11:55–64
https://doi.org/10.1007/s12602-017-9338-y

Antiaflatoxigenic Potential of Cell-Free Supernatant from *Lactobacillus plantarum* MYS44 Against *Aspergillus parasiticus*

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Published online: 24 October 2017
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Abstract The study aims to evaluate the cell-free supernatant (CFS) from *Lactobacillus plantarum* strain MYS44 against the growth and aflatoxin production by *Aspergillus parasiticus* MTCC 411. Standard in vitro techniques revealed the potential antifungal activity of CFS of *Lp*MYS44. In poison food technique, it was observed that 6% CFS of *Lp*MYS44 retarded maximum growth. The inhibition of *A. parasiticus* on peanuts confirmed the ability of CFS of *Lp*MYS44 for biopreservation. Further, CFS of *Lp*MYS44 was purified by chromatography and analyzed by GC-MS. The major antifungal compounds were oleic acid, octanoic acid, butanamide, and decanoic acid derivatives. Twofold concentrated 80 μL of CFS was found to be minimum inhibitory concentration (MIC) of CFS of *Lp*MYS44. CFS of *Lp*MYS44 suppressed the germination and growth of the spores of *A. parasiticus*. Microscopic observation showed that CFS of *Lp*MYS44 severely affected the hyphal wall of *A. parasiticus* by the leakage of cytoplasmic content leading to complete destruction. Acidic condition is favorable for CFS of *Lp*MYS44 activity. In poultry feed sample, CFS of *Lp*MYS44 reduced the aflatoxin B_1 content by 34.2%, reflecting its potentiality to use as detoxification agent. The multiple antifungal components in CFS of *Lp*MYS44 exhibited antifungal properties against aflatoxigenic *A. parasiticus* resulted in causing overall morphological changes. Furthermore, we also observed the biopreservative ability of CFS of *Lp*MYS44 against *A. parasiticus* and AFB_1 reduction in for poultry feed. This study makes a contribution to using CFS of *Lp*MYS44 and their applications in food and feed as pretreatment against aflatoxigenic *A. parasiticus* to reduce or eliminate AFB_1 and maybe other aflatoxins, produced by other *Aspergillus* spp.

Keywords CFS of *Lp*MYS44 · Aflatoxin B_1 · Antifungal metabolites · Biopreservation · Poultry feeds

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Introduction

Aflatoxins (AF) are mycotoxins that are produced by diverse *Aspergillus* species predominantly *A. flavus* and *A. parasiticus*. As secondary metabolites of these fungi, AF may contaminate a variety of food and feedstuffs, especially corn, peanuts, and cottonseed [1, 2]. The AF-contaminated food/feed causes deterioration, discoloration, and bad odors leading to severe complications in both livestock and humans, causing loss to the economy [3].

In several reports, researchers have attempted various methodologies to eliminate, inactivate, or reduce the presence of toxins in food and feedstuffs [4]. Some of the known physical and chemical strategies for detoxification of contaminated food and feed have been approved, but the use of certain chemical agents has disadvantages like nutritional value and sensory quality reduction in the processed food leading to deleterious effects on food and feed causing genotoxic and carcinogenicity [5, 6]. The use of mycotoxin binders is another way of trying to reduce the risk of mycotoxins in contaminated feed. However, these toxin binders possess a number of

disadvantages with regard to impairment of mineral utilization, narrow range of binding efficacy, etc. [7].

Therefore, there is a need for exploring safe alternatives for minimizing AF contamination in food and feed. In this regard, *Lactobacillus* with potential antifungal property would be a better choice in place of chemical preservatives for reducing the AF content in food/feed [8], as these organisms have generally been recognized as safe. They exhibit antagonistic properties by secreting various compounds possessing antifungal activity [9]. Several antifungal compounds have been isolated from supernatants of *Lactobacillus*, including lactic and acetic acids [10, 11], caproic acid [12], phenyl lactic acid, 3-hydroxylated fatty acids, and cyclic dipeptides [13]. Sangmanee and Hongpattarakere [14] identified multiple antifungal compounds secreted by *Lactobacillus plantarum* and tested their role in reducing AF production by *A. flavus*. During olive storage, application of CFS of *L. plantarum* decreased the AFB_1 levels by decreasing the quantity of molds [15]. Recently, Deepthi et al. [16] reported the potential attributes of antifungal metabolites isolated from *L. plantarum* against fumonisin-producing *Fusarium proliferatum* associated with poultry feed. However, there are limited reports on the antifungal activity of *L. plantarum* against aflatoxigenic *A. parasiticus* and its ability to reduce AF in poultry feed. Since *A. parasiticus* is the second most abundant producer of AF, there is a need to control the growth and contamination of aflatoxigenic *A. parasiticus* to overcome the problem of AF in food/feed.

In the present study, CFS from *L. plantarum* MYS44 (*Lp*MYS44) was used to find out the antifungal activity, ability to inhibit the conidial germination of *A. parasiticus*. In addition, our study also characterized the antifungal components involved in the CFS of *Lp*MYS44by GC-MS. Further, the preservative ability of CFS on peanuts was also tested, since *Aspergillus* and AF contamination is one of the major problems in the peanuts and poultry industry. The poultry feed is most vulnerable for the fungal contamination as well as mycotoxin problem, due to the use of peanuts as one of the major ingredients. Furthermore, AFB_1 reduction potential in poultry feed was also studied.

Materials and Methods

Media and Microorganisms

Aflatoxigenic *A. parasiticus* MTCC 411 strain was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. De Man Rogosa Sharpe agar (MRS agar; HiMedia, Mumbai, India) for the growth of *Lp*MYS44 and potato dextrose agar (PDA; HiMedia, India) for the growth of *A. parasiticus* was used. The conidia produced on the growth of *A. parasiticus* MTCC 411 were collected in a sterile container possessing Tween-20 (0.05%) (HiMedia, Mumbai, India). The count was adjusted to 10^6 conidia mL^{-1} using a hemocytometer.

Isolation and Characterization of *Lp*MYS44

The strain *Lp*MYS44 was isolated from the traditional fermented finger millet wine using conventional dilution and plating technique on MRS agar supplemented with 5% bromocresol purple (pH indicator). Further characterization of *Lp*MYS44 was carried out by morphological, physiological, biochemical tests and also molecular identification by 16S rRNA gene sequencing using 27F and 1492R primers as per the protocol of Rao et al. [17] (data not shown). The culture was confirmed by standard microbiological tests which were maintained in glycerol stocks at -80 °C for further use.

Preparation of CFS of *Lp*MYS44

From a stock culture, *L. plantarum MYS44* was activated by two successive subcultures in MRS broth (HiMedia, Mumbai) at 37 °C for 24 h. The final culture was obtained by adding 30 mL of the last propagation culture to 1000 mL of MRS broth (HiMedia, Mumbai) and incubating at 37 °C for 24 h. The cell concentrations achieved were established between 9 and 9.9 $\log_{10}$ CFU mL^{-1}. Then, cells were centrifuged at 5200*g* for 15 min at 4 °C. Further, the cell-free supernatant (CFS) obtained was twofold, threefold, fourfold, and sixfold concentrated at 70 °C in a Rotavapor followed by sterilization by filtration using 0.22-μm-pore-diameter membranes (HiMedia, Mumbai) and immediately used or stored at -20 °C until use within 1 month after their production.

Effect of CFS of MYS44 on Morphology of *A. parasiticus* Using Well Diffusion Assay

The antifungal activity of CFS of *Lp*MYS44 was assessed by well diffusion assay. The CFS of *Lp*MYS44 (100 μL) was poured into the wells of potato dextrose agar (HiMedia, Mumbai, India) plates swabbed with 10 μL of the conidial suspension (10^6 spores mL^{-1}), allowed CFS of *Lp*MYS44 to diffuse in the wells and incubated at 28 ± 2 °C for 5 to 7 days. The inhibitory portion of the mycelia near the zone of inhibition on the Petri plate was stacked with a cellophane tape and placed on the glass slide added with 2 to 3 drops of cotton blue stain. The morphological changes taking place in the mycelia by CFS of *Lp*MYS44 were observed under a compound microscope (×100; Carl Zeiss, Germany).

Determination of MIC by Microdilution Method

The CFS of *Lp*MYS44 was used to determine the MIC in a sterile 96-well microtiter plate. Ready-made soft agar (PDA,

100 μL) in the wells was inoculated with 10 μL of conidial suspension (10^6 spores mL^{-1}) and twofold concentrated CFS of *Lp*MYS44 (1 to 100 μL). Inoculated spore suspension in the PDA soft agar in the microtiter plate well without CFS and wells with PDA soft agar without spore suspension and CFS were served as positive and negative controls, respectively. The plate was observed for MIC, and the concentration at which fungal growth was retarded [18].

Conidial Germination Inhibition Assay

The inhibitory capability of CFS of *Lp*MYS44 on conidial germination was studied in a 24-well microtiter plate as per the procedure of Deepthi et al. [16]. The reaction consisted of 200 μL of CFS of MYS44 and 100 μL of conidia (10^6 spores mL^{-1}) mixed and made up to 1.0 mL using phosphate buffered saline (PBS). Control was 100 μL of *A. parasiticus* (10^6 spores mL^{-1}) made up to 1.0 mL using PBS. The microtiter plate was incubated for 24 h at 28 ± 2 °C. Conidial germination at time intervals of 2, 4, 6, and 8 h was observed by staining the reacted sample with acridine orange (HiMedia, Mumbai, India) under a fluorescent microscope (Carl Zeiss, Germany). Germinated conidia were counted using hemocytometer. Conidia germination percent was calculated using the formula: [no. of conidia germinated / total conidia counted] × 100.

Effect of Different pH Treatments on CFS of *Lp*MYS44 Activity

The inhibitory effect of CFS of *Lp*MYS44 was assessed after treatment at pH levels of 3.0, 4.0, 5.0, and 7.0 ± 0.2. *A. parasiticus* spore suspension (10^6 spores mL^{-1}) was dispensed to the media and the activity at each pH level was calculated as percent inhibition of *A. parasiticus* [19]. The MRS media with pH 3.0, pH 4.0, pH 5.0, and pH 7.0 devoid of CFS was served as the control for test of each pH. The media without pH treatment served as negative control. The inhibition percentage was calculated as follows:

$$I\ (\%) = [(C - T)/C] \times 100$$

I % = percent inhibition; C = growth diameter in control; T = growth diameter in pH-treated group.

Extraction and GC-MS Analysis of CFS of *Lp*MYS44 for Potential Antifungal Metabolites

The CFS was collected from the freshly grown culture of *Lp*MYS44 by centrifuging at 10,000 rpm for 30 min and extracted with ethyl acetate (1:3). Crude compound was purified using combined hexane/ethyl acetate solvent system and silica gel mini-column chromatography. The active fraction of the crude extract was initially rectified by thin-layer chromatography and analyzed using GC-MS at the sophisticated analytical instrumentation facility (IIT, Madras, India) with a TR-Wax MS column (length 30 m, film thickness 0.25 mm, ID 0.25 mm). The inlet temperature was 250 °C with the split ratio 10:1. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The active fraction of the compound was maintained at 40 °C for 2 min. The temperature was increased at the rate of 6 °C/min to 230 °C and held for 5 min. For MS detection, an electron ionization mode was used with ionization energy of 70 eV, ion source temperature of 245 °C, and scan mass range of 35–500 amu. The bioactive components were identified based on the comparison of their relative retention times and fragmentation patterns of mass spectra [18].

Quantification of AFB1 in Poultry Feed Treated with CFS of *Lp*MYS44

Aspergillus and AF contamination is one of the major problems in the poultry industry. The poultry feed is most vulnerable for the fungal contamination as well as mycotoxin problem, due to the use of peanuts as one of the major ingredients. In view of this, we have selected both peanuts and poultry feed as a model system. Autoclaved poultry feed (10 g) was used in each of the following three groups: (1) positive control: feed inoculated with 50 μL (10^6 spores mL^{-1}) of *A. parasiticus* spore suspension (poultry feed + *A. parasiticus*); (2) negative control: feed inoculated with 50 μL of MRS broth and 50 μL (10^6 spores mL^{-1}) of *A. parasiticus* spore suspension (poultry feed + *A. parasiticus* + MRS broth); and (3) test: feed inoculated with 50 μL of CFS of *Lp*MYS44and 50 μL (10^6 spores mL^{-1}) of *A. parasiticus* spore suspension (poultry feed + *A. parasiticus* + CFS of *Lp*MYS44). Inoculated plates were incubated at 28 ± 2 °C for 21 days. AFB_1 determination and its quantification were done by a solvent extraction method, TLC (HiMedia, Mumbai, India) and UV spectrophotometer (Thermo Scientific) analysis. After 21 days of incubation, the inoculated feed samples from respective treatment were extracted with an equal volume (1:1) of chloroform. The organic phase obtained was pooled and concentrated using a rotary evaporator (Rotavapor, R300, India) at 350 bars and 50 °C. AFB_1 in each treatment was detected on HPTLC plates (HiMedia, India) using the mobile phase chloroform:ethyl acetate (8:2) and observed under UV. Further, the fraction showing either blue or green band was pooled and concentrated as explained earlier. For further confirmation, the suspected AFB_1 fraction was separated on HPTLC plates along with standard (AFB_1 standard, procured from Sigma-Aldrich, India). The suspected AFB_1 fraction was subjected to UV-visible spectra ranging from 200 to 600 nm. Spectra were analyzed for the presence of peak at 350 nm which confirms the presence of AFB_1 in the sample. The concentration of AF

in the samples was determined by using UV spectroscopy. A true analytical concentration of particular mycotoxin was measured as outlined in the Association of Official Analytical Chemists (AOAC) 16th Edition, Methods of Analysis. The absorbance at maximum wavelength was measured and compared to molar absorptivity and thus the concentration of AFB_1 was determined as follows:

$$\mu g/mL\ AFB1 = \frac{Absorbance \times Molecular\ weight \times 1000}{Molar\ Absorptivity}$$

Percent Reduction (%) of AFB_1 in the feed samples was calculated as,

$$\%Reduction = 1 - T/C \times 100$$

T = AFB_1concentration in treated sample; C = AFB_1 concentration in positive control.

Biopreservative Efficacy of CFS of *Lp*MYS44on Peanuts

The assay was carried out as per the procedure of Yang and Chang [8]. Peanuts (10 g) were soaked for 5 h in 100 mL distilled water, autoclaved and the cooked peanuts were soaked in the CFS of *Lp*MYS44 at pH 3.5 ± 0.2 for 8 h at room temperature and transferred to sterile Petri dishes. Ten microliters of the conidial suspension (10^6 spores mL^{-1}) was inoculated on each peanut seed. The assay was divided into four groups comprising of (1) control-inoculated with *A. parasiticus* and MRS broth, (2) peanuts treated with both CFS and *A. parasiticus*, (3) peanuts treated with CFS alone, and (4) a group with untreated peanuts and the mycelial growth was observed each day up to 6 days.

Statistical Analysis

Three independent replicates of each experiment were obtained. The results were expressed as significant differences of mean, standard deviation, and graphical representation using one-way ANOVA followed by Tukey's post hoc test using GraphPad prism version 5.03.

Results

Antifungal Inhibitory Spectrum of CFS of *Lp*MYS44

The CFS of *Lp*MYS44 assayed for antifungal activity in well diffusion plates revealed that 100 μL of crude extract effectively controlled *A. parasiticus* growth, which is comparable to the standard nystatin (Fig. 1). In the MIC test, 80 μL of twofold CFS of *Lp*MYS44 was considered as a minimal inhibitor based on the visible restriction of fungal growth (Fig. 2). Microscopic examination revealed that the mycelia were severely affected by CFS of *Lp*MYS44 which is evident by the damage caused to the hyphal wall and subsequent leakage of cytoplasmic contents resulting in complete destruction (Fig. 3).

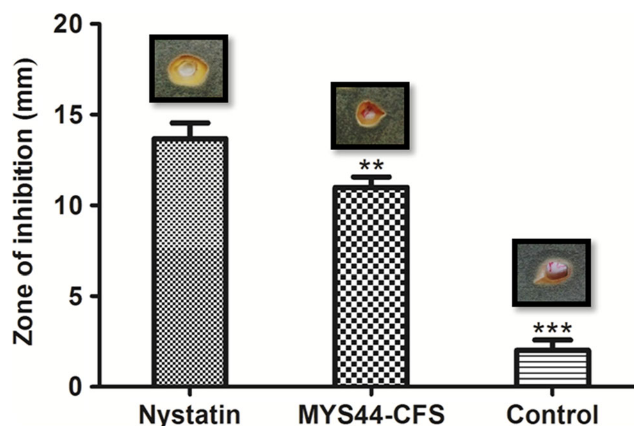

Fig. 1 Inhibitory effect of CFS of MYS44 by well diffusion assay

Effect of pH on Antifungal Activity of CFS of *Lp*MYS44

The antifungal activity of CFS of *Lp*MYS44 examined at different pH levels revealed that the inhibitory activity was reduced from 72 to 22% on neutralization, as compared to 30.65% for negative control (Fig. 4).

A. parasiticus Conidial Germination Inhibition Assay

The germination of conidia and mycelial growth were severely affected by CFS of *Lp*MYS44. Based on the microscopic examination, there was a delay in germination of conidia in treated samples (*A. parasiticus* (10^6 spores/mL) + CFS of *Lp*MYS44), and elongation of most of the germ tubes was hampered and did not grow (Fig. 5). However, 6.74% of conidial germination was observed at 6 h of incubation. The control group (*A. parasiticus*; 10^6 spores/mL) developed germ tube from the apical cells after 6 h of incubation with 44.2% conidial germination (Table 1). Due to the presence of

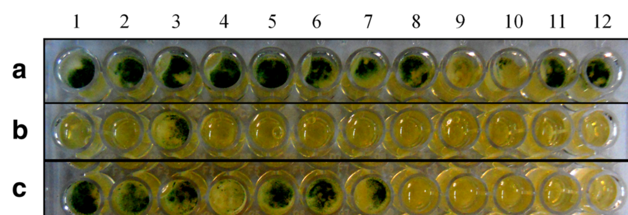

Fig. 2 Determination of MIC of CFS of MYS44. **a** Positive control (*Aspergillus parasiticus*, 10^6 spores/mL—10 to 100 μL). **b** Negative control (uninoculated PDA soft agar). **c** Twofold dilution of CFS of MYS44 (10 to 100 μL); 80 μL of twofold CFS showing visible inhibition in well C8 indicates MIC

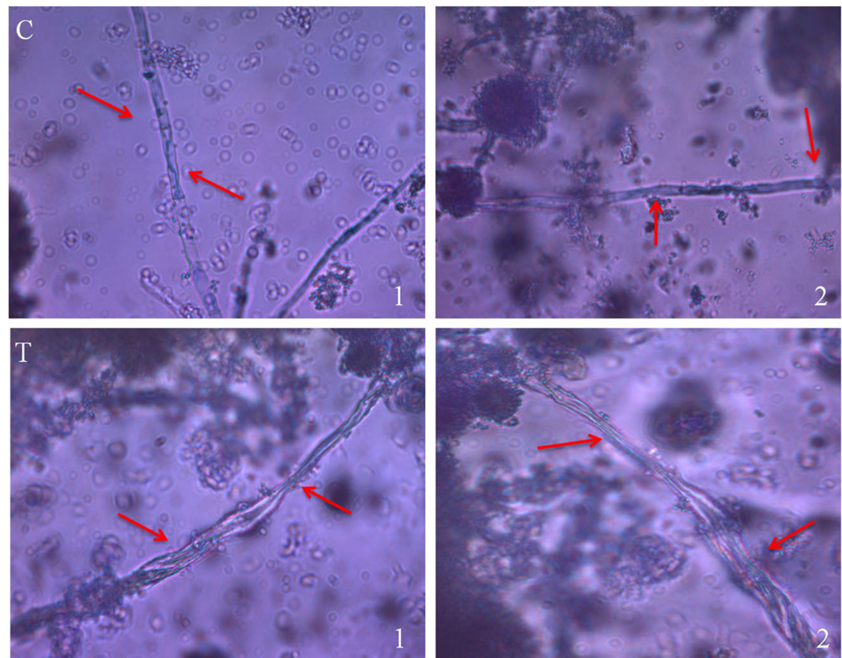

Fig. 3. Compound micrograph images showing the effects of CFS of MYS44 on *Aspergillus parasiticus*. (C-1, C-2) Control, *A. parasiticus* appears to be smooth-surfaced, even width, tubular hyphae. (T-1, T-2) *A. parasiticus* treated with CFS of MYS44 showing disrupted and shrunken hyphae

bioactive metabolites in CFS of *Lp*MYS44 in treated sample, the unfavorable environment caused disruption in conidial germination.

Characterization of Potential Antifungal Metabolites

The GC-MS analysis of filter sterilized CFS of *Lp*MYS44 in our study revealed 15 bioactive compounds among which 10 showed 100% base rate (Fig. 6A–B), which included butanamide, 2-hydroxy-*N*,3,3 trimethyl, octanoic acid, undecanoicacid-10-methyl-methyl ester, dodecanoic acid, tridecanoicacid-12-methyl-, methyl ester, tetradecanoic acid, hexadecanoicacid-methyl ester, *n*-hexadecanoic acid, 8-octadecanoic acid methyl ester and oleic acid (Table 2). However, docosanoic acid, methyl ester was found to be least base rate (51.8%). Oleic acid was the abundant antifungal component produced by *Lp*MYS44.

Effect of CFS of MYS44 on AFB1 in Poultry Feed

Great efforts have been put in either to eliminate AFB_1 completely or to reduce its content to significantly lower level in feed as the AFB_1 is a potent source of health hazard to poultry and human. In the present study, poultry feed inoculated with *A. parasiticus* and CFS of *Lp*MYS44 has shown to significantly reduce (37.5 μg/mL (34.2%)) AFB_1 concentration compared to AFB_1 concentration (57 μg/mL) in control sample (Fig. 7). The high reduction of AFB_1 content in the CFS of *Lp*MYS44-treated group may be due to the presence of antiaflatoxigenic metabolites produced by *Lp*MYS44 during the growth and development.

Biopreservative Ability of CFS of *Lp*MYS44 on Peanuts

As peanut forms the main constituent in animal feed and highly susceptible to *Aspergillus* contamination and AF production, edible peanuts were chosen as an experimental model to assess the preservative ability of *Lactobacillus*. In our current investigation, we found that the fungal growth of *A. parasiticus* was completely reduced in twofold-treated CFS group as compared to control after 6 days of incubation, suggesting that the CFS exhibited an inhibitory activity to control fungal growth on peanuts (Fig. 8). Controlling the

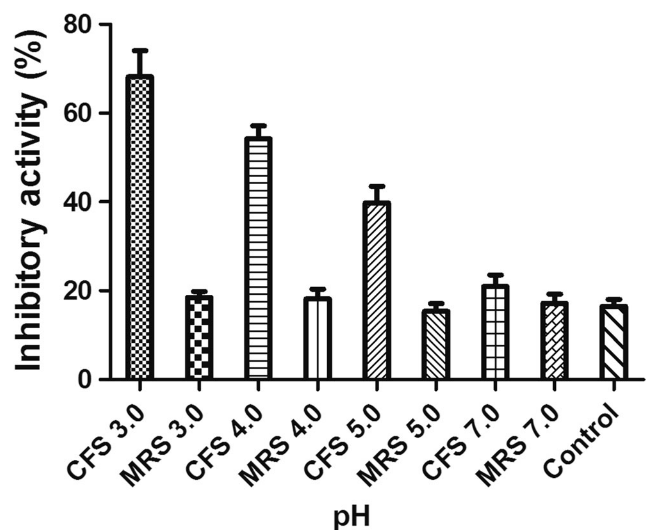

Fig. 4 Effect of different pH treatments on the inhibitory potential of CFS of MYS44. The data represents mean ± S.D. of three representative pH values. CFS, cell-free supernatant of *Lp*MYS44; MRS, sterile de Man Rogsa Sharpe broth

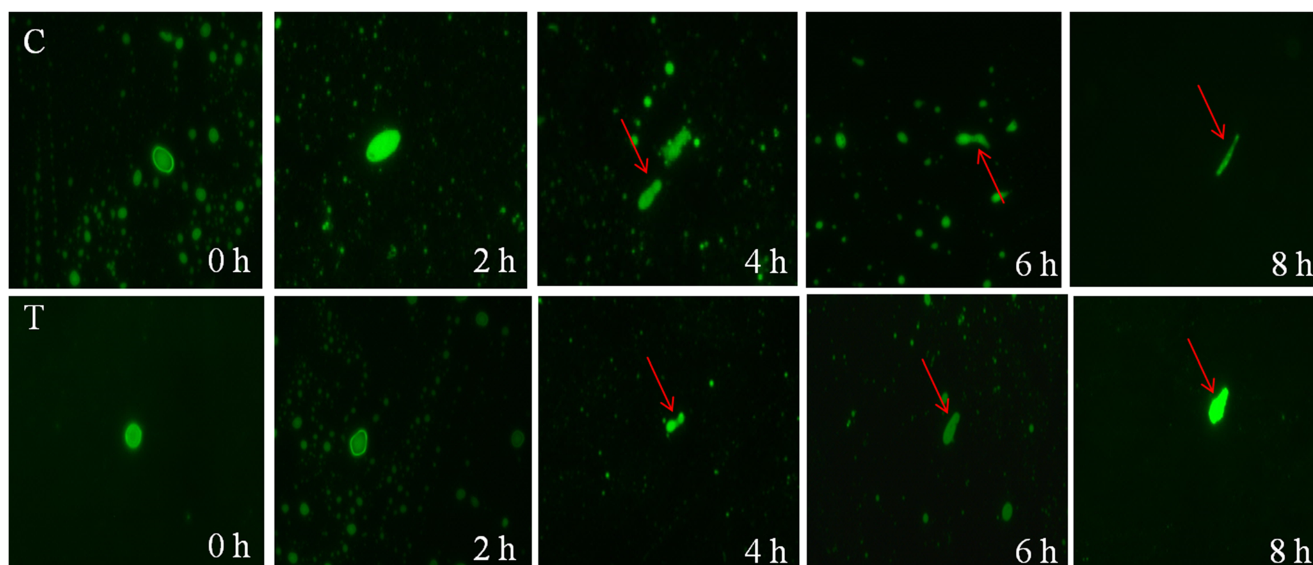

Fig. 5 Spore germination inhibition of *A. parasiticus* by CFS of MYS44. (C) Control, *A. parasiticus* showing germtube initiation and formation at 6 and 8 h of incubation, respectively. (T) Treated, *A. parasiticus* treated with CFS of MYS44 showing retarded germination and germ tube formation. Images obtained by fluorescent microscopy at ×1000 magnification

growth of *A. parasiticus* on peanuts reduces the fungal contamination during storage and also facilitates to use of better raw materials.

Discussion

The antifungal activities of *Lactobacillus* strains have been reported by previous studies. Ilavenil et al. [20] observed a broad-spectrum antifungal property of *Lactobacillus* strains against *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Penicillium roqueforti*, *Botrytis elliptica*, and *Fusarium oxysporum*. Thus, the present work reports on the efficacy of the CFS of *Lp*MYS44 having multiple antifungal components that was able to demonstrate the antifungal activity and their ability to reduce or eliminate AFB_1.

Similarly, metabolites of *L. plantarum* exhibited antifungal activity against bakery spoilage fungi such as, *Gibberella moniliformis* and *A. fumigatus* [21]. The microbial community composition along with environmental and growth conditions are the main factors for their antifungal activity [22]. According to Ilavenil et al. [20], the primary and secondary metabolites produced by *Lactobacillus* act as strong antagonistic agents and as microbial inhibitors. In the present study, we have used *L. plantarum* MYS44 isolated from finger millet (*Eleusine coracana*) wine. The morphological, physiological, biochemical, and molecular studies have confirmed its identification. Recently, Deepthi et al. [16] isolated multiple antifungal compounds from *L. plantarum* strain MYS6 which had inhibitory activity against fumonisin-producing *F. proliferatum* associated with poultry feed. The phylogenetic relationship between *L. plantarum* MYS44 and *L. plantarum* MYS6 has been represented in Fig. 9.

Table 1 Conidial germination inhibition by CFS of MYS44

Time (h)	% germination of conidia	
	A. p	*A. p* + CFS of MYS44
2	0.0	0.0
4	29.67 ± 2.11*	0.0
6	44.23 ± 3.42**	6.74 ± 1.21**
8	62.92 ± 3.86*	13.69 ± 1.52*

Mean ± SD; *A.p*, *Aspergillus parasiticus*; CFS of MYS44, cell-free supernatant of *Lactobacillus plantarum* MYS44

Significant differences among the groups: $*P < 0.1$, $**P < 0.01$; one-way ANOVA followed by post hoc Tukey's test)

The inhibitory compounds were mainly organic acids comprising of lactic acid, oleic acid, octanoic acid, etc., which act as protective weapons against spoilage fungi. Though the isolates (*Lp*MYS44 used in our study and *Lp*MYS6 used by Deepthi et al. [16]) contribute to the antifungal activity against *A. parasiticus* and *F. proliferatum*, respectively, the isolate *Lp*MYS44 shares some similarities and differences in the morphological, biochemical, and molecular identification features compared to isolate *Lp*MYS6. For instance, the isolate *Lp*MYS44 used in the present study exhibited similar morphological features like Gram positive, catalase negative, homo-fermentative, and positive for arginine hydrolysis. The divergent features include the isolate *Lp*MYS44 that was from a different isolation source, able to ferment sorbitol, and unable to ferment arabinose, which are in contrast to the features of isolate *Lp*MYS6. The phylogenetic analysis based on the 16S rDNA sequence showed that both isolates were in

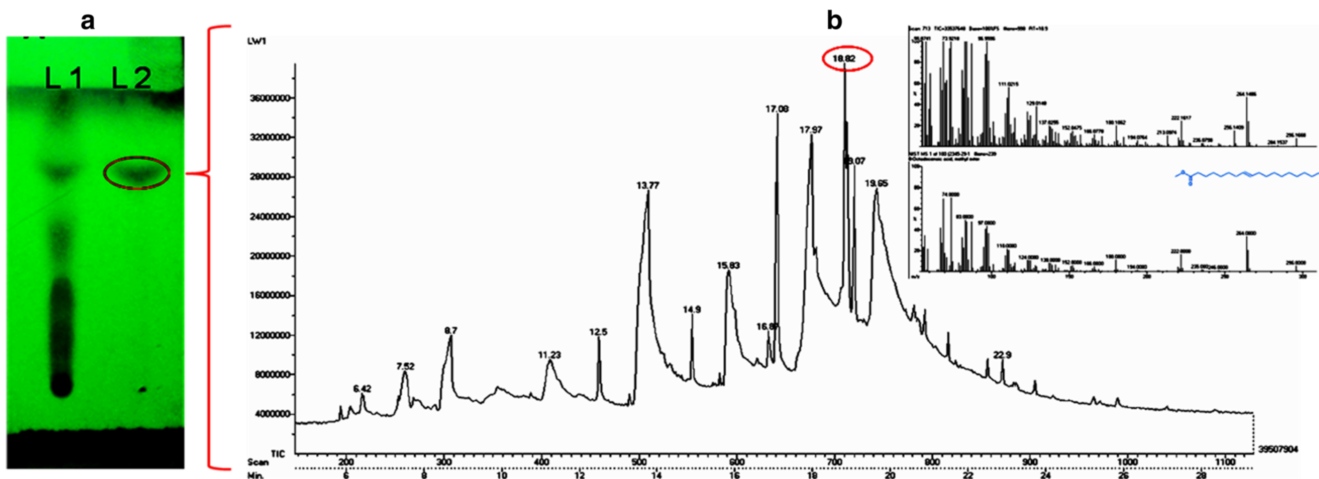

Fig. 6 TLC followed by GC-MS analysis of the purified antifungal compound of *Lp*MYS44 (**a** and **b**). L1, crude extract; L2, purified compound

different nodes. In the present study, the secondary metabolites produced by *Lp*MYS44 exhibited strong antifungal activity against *A. parasiticus*, which is the second major producer of AFB_1.

The antifungal activity may not only be due to low pH in situ, but possibly, the secretion of antifungal organic metabolites also might have contributed. It is well known that *Lactobacillus* produces secondary metabolite acids like acetic and lactic or special acids. The ideal pH of the organic acids to penetrate the microbial cell is beneath their p*Ka* values. The p*Ka* values of common organic acids produced by *Lactobacillus* are below 5.0 [23]. Thus, in the present study, when the pH of CFS is above 5.0, the organic acids may not exhibit antifungal activity. Neutralization of CFS lowers the antifungal activity and this fact strongly suggests that the organic acids are responsible for the antifungal activity. Sangmanee and Hongpattarakere [14] reported that the activity of CFS of *L. plantarum* K35 was optimum at pH 4.0 but it

Table 2 Identified antifungal compounds in CFS of *Lactobacillus plantarum* MYS44

Compound	Molecular weight	Molecular Formula	Structure	PubChem CID	Retention Time	% Base Peak
p-Dioxane-2,3-diol	120.1039	$C_4H_8O_4$		96170	6.42	88.5
Butanamide,2-hydroxy-N,3,3 trimethyl	159.1124	$C_8H_{17}NO_2$		-	12.57	100
Octanoic acid	144.2114	$C_8H_{16}O_2$		379	8.7	100
n-Decanoic acid	172.2646	$C_{10}H_{20}O_2$		2969	11.23	77.3
Undecanoic acid,10-methyl-,methyl ester	214.3443	$C_{13}H_{26}O_2$		-	12.5	100
Dodecanoic acid	200.3178	$C_{12}H_{24}O_2$		3893	13.77	100
Tridecanoic acid, 12-methyl-, methyl ester	242.3975	$C_{15}H_{30}O_2$		15608	14.9	100
Tetradecanoic acid	228.3709	$C_{14}H_{28}O_2$		15608	15.83	100
9-hexadecanoic acid, methyl ester	268.4348	$C_{17}H_{32}O_2$		643801	16.87	64.1
Hexadecanoic acid, methyl ester	270.4507	$C_{17}H_{34}O_2$		8181	17.12	100
n- Hexadecanoic acid	256.4241	$C_{16}H_{32}O_2$		985	18.02	100
8-Octadecanoic acid, methyl ester	296.4879	$C_{19}H_{36}O_2$		5364422	18.9	100
Heptadecanoic acid, 16-methyl, methyl ester	298.5038	$C_{19}H_{38}O_2$		-	19.1	78.3
Oleic acid	282.4614	$C_{18}H_{34}O_2$		445639	19.65	100
Docosanoic acid, methyl ester	354.6101	$C_{23}H_{46}O_2$		13584	22.9	51.8

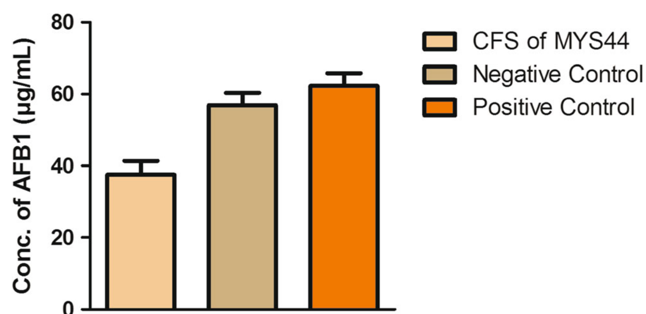

Fig. 7 Aflatoxin reduction ability of CFS of MYS44 in poultry feed. The data represents mean ± S.D. of three representative values of CFS of MYS44 and control

rapidly decreased after neutralization, thereby supporting the present finding.

Several investigators have previously reported the interactions of CFS of *Lactobacillus* against the fungal conidial germination [24, 25]. In one study, the addition of concentrated CFS from *L. reuteri* R2 inhibited the germ tube formation and successive hyphal development in *Trichophyton tonsurans* [26]. Similarly, disruption and lysis of cell wall in *Penicillium citrinum* cell wall and reduction in hyphal wall branching were observed after treatment with brevicin SG1 produced by *L. brevis* SG1 [27]. Treatment of *A. parasiticus* MTCC 2796 with an unidentified antifungal compound from *Pediococcus acidolactici* LAB 5 produced undulating mycelial cell surface of mycelia and size reduction in conidial structures [28]. Our findings in morphological changes on conidial germination of *A. parasiticus* are consistent with the findings of Crowley et al. [25], who reported that the inhibition of spores, germ tubes, and hyphae of *A. fumigatus* Af293 was treated with unidentified antifungal compounds of *L. plantarum* [16].

In earlier studies, oleic acid has been shown to possess the potential inhibitory component against plant pathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avenae*, and *Crinipellis perniciosa* [29]. In addition, Corcoran et al. [30] also demonstrated that the presence of oleic acid enhances the growth of *Lactobacilli* and their survival in gastric juice and Al-Naseri et al. [31] also reported that octanoic acid has a key role in lactose and galactose catabolic pathways of nonstarter lactic acid bacteria. Three antifungal substances such as cyclo-(Leu-Pro), 2,6-diphenyl-piperidine, and 5,10-diethoxy-2,3,7,8-tetrahydro-1 H, 6H-dipyroll [1,2-a; 1′, 2′-d] pyrazine were identified in some other *Lactobacillus* species but other than *L. plantarum*. They further established that octanoic acid is the synthetic acid name for the eight-carbon saturated fatty acid known by caprylic acid. In another study, Jiang et al. [32] reported the production of short chain diols such as dioxane derivatives as antifungal metabolites from *Lactobacillus* sp. On the other hand, Sjogren et al. [33] identified decanoic acid derivatives with antifungal properties in *L. plantarum* Mi LAB 14. As per these studies and several others, the identified antifungal compounds in the current study reflect the diversity and wide distribution among different strains of *L. plantarum* and different *Lactobacillus* sp. Our investigation also indicates that multiple bioactive compounds in CFS of *Lp*MYS44 may have a significant contribution to the antifungal inhibitory potential against *A. parasiticus*, and furthermore, production of multiple antifungal compounds by *Lp*MYS44 indicates a broad-spectrum inhibitory potential against aflatoxigenic *A. parasiticus*.

In support of our observation, Gourama et al. [34] also reported the presence of a metabolite that inhibited the accumulation of AF in cell-free extract of *Lactobacillus*. Karunaratne et al. [35] stated that the reduction in AF content was not due to the production of hydrogen peroxide or decrease in pH of the media but probably due to some specific antifungal metabolites produced by *Lactobacillus*. In addition, researchers have reported that the genus *Lactobacillus* are the better detoxifiers of AF than *Pediococcus* and *Leuconostoc* [36]. The detoxifying ability of *Lactobacillus* primarily depends on the activity of secondary metabolites that degrade toxins to less toxic or non-toxic forms. In a study by Sangmanee and Hongpattarakere [14], supernatant from

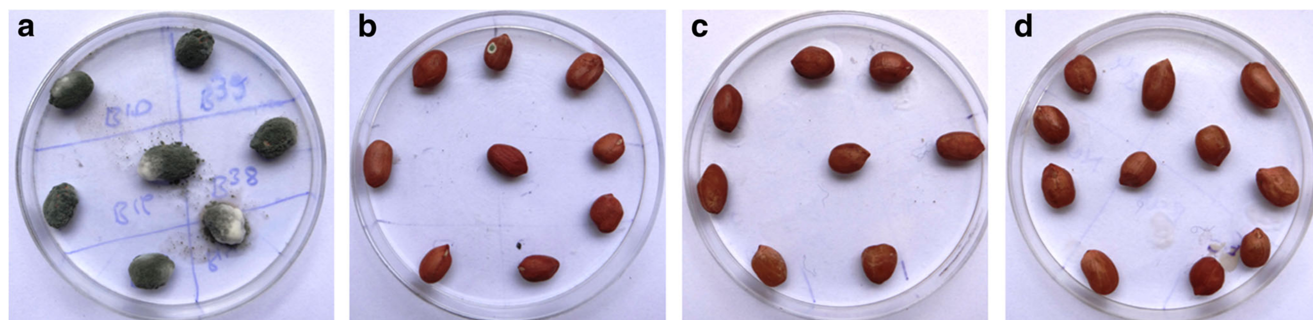

Fig. 8 Biopreservative efficacy of CFS of MYS44 on peanuts. Incubation after 6 days of incubation, peanuts treated with MRS broth and *A. parasiticus* in control showing presence of spores (**a**), complete inhibition on peanuts treated with CFS and *A. parasiticus* spores (**b**), CFS alone treated peanuts (**c**), untreated peanuts in the control group (**d**), respectively, showing no growth of *A. parasiticus*

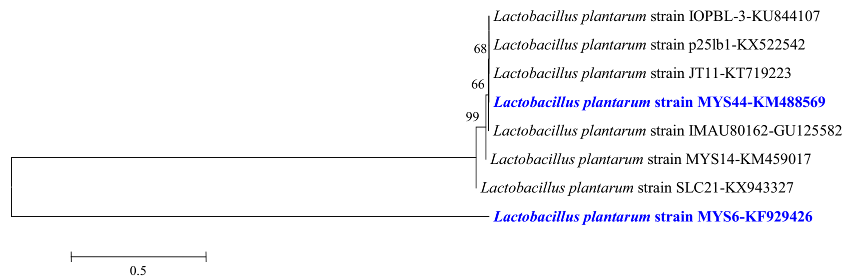

Fig. 9 Phylogenetic relationships obtained by dendrogram based on BLAST algorithm and neighbor-joining tree method between *Lactobacillus plantarum* MYS44 and *Lactobacillus plantarum* MYS06 and other *Lactobacillus plantarum* strains

L. plantarum was observed to reduce AF content by 36.77% in the growth medium. Similarly, Deepthi et al. [16] also reported the fumonisin detoxification ability of *L. plantarum* strain MYS6 using poultry feed as a model. There are very limited reports on the reduction of AFB_1 by the supernatant of *Lactobacillus* using poultry feed model. Thus, the findings of our study indicate that the presence of multiple inhibitory components of CFS of *Lp*MYS44 in poultry feed may hinder the growth and development of *A. parasiticus* and production of AFB_1 by *A. parasiticus*. However, the detoxification mechanism reported here needs to be exploited further for practical application of CFS of *Lp*MYS44 in poultry feeds.

The antagonistic properties of CFS of *Lactobacillus* against *Aspergillus* species have been reported in earlier studies. Kumar et al. [37] confirmed the inhibitory potential of *Bacillus cereus* against *A. flavus* and *A. niger* in the process of bakery food spoilage. In addition, Yang and Chang [8] reported the biopreservative efficacy of *L. plantarum* against *A. flavus* using soybean model and they found that the preservative property of *L. plantarum* was due to cyclo (Leu-Leu) present in the supernatant of cell culture. Rather et al. [11] who also studied *L. plantarum*-soya bean model reported that the fivefold concentrated CFS of *L. plantarum* YML007 completely inhibited the growth of *A. niger*. Hence, we also conclude as per the data we obtained in our study that the presence of inhibitory components in the CFS of *Lp*MYS44 might have played a crucial role in controlling the *A. parasiticus* in peanuts, which forms an important component of poultry feed.

In conclusion, our present investigation showed that the multiple antifungal components with CFS of *Lp*MYS44 exhibited antifungal properties against aflatoxigenic *A. parasiticus*. The antifungal property of these components resulted in causing overall morphological changes in *A. parasiticus*, destroyed the hyphal wall, and inhibited conidial germination. In addition, using chromatographic analysis, antifungal property of CFS of *Lp*MYS44 is witnessed by AFB_1 reduction in *A. parasiticus.* Furthermore, we also observed the biopreservative ability of CFS of *Lp*MYS44 against *A. parasiticus* and AFB_1 reduction in poultry feed. Thus, we conclude that the CFS of *Lp*MYS44 may be further explored for their antifungal properties and their applications in food and feed as pretreatment against aflatoxigenic *A. parasiticus* to reduce or eliminate AFB_1 and maybe other AF produced by other *Aspergillus* sp.

Acknowledgments We thank the Central Instrumentation Facility, Institute of Excellence (IOE), University of Mysore for providing timely assistance for fluorescent microscopy. We also thank the SERB (Science and Engineering Research Board), India (No. EEQ/2016/000273; dated 23/02/2017) for their valuable support.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

References

1. Yu J, Whitelaw C, NiermanWC BD, Cleveland TE (2004) *Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. FEMS Microbiol Lett 237:333–340
2. Rodrigues P, Venâncio A, Kozakiewicz Z, Lima N (2009) A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section Flavi isolated from Portuguese almonds. Int J Food Microbiol 129:187–193
3. Gerbaldo G, Barberis C, Pascual L, Dalcero A, Barberis L (2012) Antifungal activity of two *Lactobacillus* strains with potential probiotic properties. FEMS Microbiol Lett 332:27–33
4. Khanafari A, Soudi H, Miraboulfathi M (2007) Biocontrol of *Aspergillus flavus* and aflatoxin B1 production in corn. Iran J Environ Health Sci Eng 4:163–168
5. Guan D, Li P, Zhang Q, Zhang W, Zhang D, Jiang J (2011) An ultra-sensitive monoclonal antibody-based competitive enzyme immunoassay for aflatoxin M1 in milk and infant milk products. Food Chem 125:1359–1364
6. El-aziz ARMA, Mahmoud MA, Al-othman MR, Al-gahtani MF (2015) Use of selected essential oils to control aflatoxin contaminated stored cashew and detection of aflatoxin biosynthesis gene. Sci World J 1–13
7. Huwig A, Freimund S, Käppeli O, Dutler H (2001) Mycotoxin detoxication of animal feed by different adsorbents. Toxicol Lett 122(2):179–188
8. Yang EJ, Chang HC (2010) Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. Int J Food Microbiol 139:56–63
9. Wang H, Yan H, Shin J, Huang L, Zhang H, Qi W (2011) Activity against plant pathogenic fungi of *Lactobacillus plantarum*

IMAU10014 isolated from Xinjiang koumiss in China. Ann Microbiol 61:879–885
10. Dalié DK, Deschamps AM, Atanasova-Penichon V, Richard-Forget F (2010) Potential of *Pediococcus pentosaceus* (L006) isolated from maize leaf to suppress fumonisin-producing fungal growth. J Food Prot 73(6):1129–1137
11. Ahmad Rather I, Seo BJ, Rejish Kumar VJ, Choi UH, Choi KH, Lim JH, Park YH (2013) Isolation and characterization of a proteinaceous antifungal compound from *Lactobacillus plantarum* YML007 and its application as a food preservative. Lett Appl Microbiol 57(1):69–76
12. Corsetti A, Gobbetti M, Rossi J, Damiani P (1998) Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. Appl Microbiol Biotechnol 50(2):253–256
13. Strom K, Sjogren J, Broberg A, Schnurer J (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 68(9):4322–4327
14. Sangmanee P, Hongpattarakere T (2014) Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. Food Control 40: 224–233
15. Kachouri F, Ksontini H, Hamdi M (2014) Removal of aflatoxin B1 and inhibition of *Aspergillusflavus* growth by the use of *Lactobacillus plantarum* on olives. J Food Prot 77:1760–1767
16. Deepthi BV, Rao KP, Chennapa G, Naik MK, Chandrashekara KT, Sreenivasa MY (2016) Antifungal attributes of *Lactobacillus plantarum* MYS6 against fumonisin producing *Fusarium proliferatum* associated with poultry feeds. PloS One 11(6): e0155122
17. Poornachandra Rao K, Chennappa G, Suraj U, Nagaraja H, Charith Raj AP, Sreenivasa MY (2015) Probiotic potential of *Lactobacillus* strains isolated from sorghum-based traditional fermented food. Probiotics Antimicrob Proteins 7(2):146–156
18. Gerez CL, Torres MJ, Font de Valdez G, Rollán G (2013) Control of spoilage fungi by lactic acid bacteria. Biol Control 64:231–237
19. Wang H, Yan Y, Wang J, Zhang H, Qi W (2012) Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. PLoS One 7:e29452
20. Ilavenil S, Park HS, Vijayakumar M, Valan Arasu M, Kim DH, Ravikumar S, Choi KC (2015) Probiotic potential of *Lactobacillus* strains with antifungal activity isolated from animal manure. Sci World J
21. Arasu MV, Kim DH, Kim PI, Jung MW, Ilavenil S, Jane M, Choi KC (2014) In vitro antifungal, probiotic and antioxidant properties of novel *Lactobacillus plantarum* K46 isolated from fermented sesame leaf. Ann Microbiol 64(3):1333–1346
22. Schillinger U, Villarreal JV (2010) Inhibition of *Penicillium nordicum*in MRS medium by lactic acid bacteria isolated from foods. Food Control 21(2):107–111
23. De Muynck C, Leroy AI, De Maeseneire S, Arnaut F, Soetaert W, Vandamme EJ (2004) Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. Microbiol Res 159(4):339–346
24. Mauch A, Dal Bello F, Coffey A, Arendt EK (2010) The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. Int J Food Microbiol 141(1):116–121
25. Crowley S, Mahony J, van Sinderen D (2013) Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends Food Sci Technol 33(2):93–109
26. Guo J, Mauch A, Galle S, Murphy P, Arendt EK, Coffey A (2011) Inhibition of growth of *Trichophyton tonsurans* by *Lactobacillus reuteri*. J Appl Microbiol 111(2):474–483
27. Adebayo CO, Aderiye BI (2011) Suspected mode of antimycotic action of brevicin SG1 against *Candida albicans* and *Penicillium citrinum*. Food Control 22(12):1814–1820
28. Mandal V, Sen SK, Mandal NC (2013) Production and partial characterization of an inducer-dependent novel antifungal compound(s) by *Pediococcus acidilactici* LAB 5. J Sci Food Agric 93(10):2445–2453
29. Walters D, Raynor L, Mitchell A, Walker R, Walker K (2004) Antifungal activities of four fatty acids against plant pathogenic fungi. Mycopathologia 157:87–90
30. Corcoran BM, Stanton C, Fitzgerald GF, Ross RP (2007) Growth of probiotic *Lactobacilli* in the presence of oleic acid enhances subsequent survival in gastric juice. Microbiology 153:291–299
31. Al-Naseri A, Bowman JP, Wilson R, Nilsson RE, Britz ML (2013) Impact of lactose starvation on the physiology of *Lactobacillus casei* GCRL163 in the presence or absence of tween 80. J Proteome Res 12:5313–5322
32. Jiang Y, Liu W, Zou H, Cheng T, Tian N, Xian M (2014) Microbial production of short-chain diols. Microb Cell Factories 13(1):165
33. Sjogren J, Magnusson J, Broberg A, Schnürer J, Kenne L (2003) Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. Appl Environ Microbiol 69(12):7554–7557
34. Gourama H, Bullerman LB (1995) Antimycotic and antiaflatoxigenic effect of lactic acid bacteria: a review. J Food Prot 58(11):1275–1280
35. Karunaratne A, WezenbergE BLB (1990) Inhibition of mold growth and aflatoxin production by *Lactobacillus* sp. J Food Prot 53(3):230–236
36. Zinedine A, Faid M, Benlemlih M (2005) *In vitro* reduction of aflatoxin B1 by strains of lactic acid bacteria isolated from Moroccan sourdough bread. Int J Agric Biol 7:67–70
37. Kumar SN, Sreekala SR, Chandrasekaran D, Nambisan B, Anto RJ (2014) Biocontrol of *Aspergillus* species on peanut kernels by antifungal diketopiperazine producing *Bacillus cereus* associated with entomopathogenic nematode. PLoS One 9(8):e106041