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Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*

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ABSTRACT

The antifungal activity of *Lactobacillus plantarum* K35 isolated from traditional Thai fermented rice noodle was evaluated against the growth and aflatoxin production of *Aspergillus flavus* TISTR304 and *Aspergillus parasiticus* TISTR3276. Multiple antifungal compounds secreted by *L. plantarum* were firstly analyzed using GC–MS in parallel to the ultrastructure alteration of the treated fungi. *L. plantarum* K35 supernatant caused significant reduction of the fungal growth and aflatoxin production. The antifungal activity was pH-dependent and favorable to acidic conditions whereas the catalase treatment had no influence indicating no involvement of hydrogen peroxide in the inhibition. Antifungal substances were resistant to sterilization and proteolytic enzymes including trypsin and proteinase K. The major components, apart from lactic acid, were 2-butyl-4-hexyloctahydro-1H-indene (19.55%), oleic acid (10.52%) and palmitic acid (7.27%). Other minor antifungal compounds included linoleic acid (2.11%), 2,4-di-*tert*-butylphenol (1.84%), stearic acid (1.55%), 3-phenyllactic acid (1.42%) and pyroglutamic acid (1.07%). The morphological changes of the fungi exposed to the supernatant were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *L. plantarum* K35 supernatant caused severe damage to the cell wall and cytoplasmic membrane leading to a massive loss of cytoplasmic content, the formation of membrane-bound vesicles, and complete destruction of membranous organelles including mitochondria and nucleus.

1. Introduction

Aflatoxins are secondary metabolites, which are acutely and chronically toxic to humans and animals. The major naturally produced ones are aflatoxin B_1, B_2, G_1 and G_2. They cause acute liver damage, liver cirrhosis, the induction of tumors and teratogenic effects. Certain strains of the fungi *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* were reported to produce aflatoxins (Angsubhakorn, 1989; Egal et al., 2005). These ubiquitous fungi are widely distributed in nature, and responsible for the deterioration of foods as well as agricultural produces. Various preservation methods using physical, chemical and biological strategies have been applied to extend the shelf-life and increase safety in foods and feeds (Aziz, El-Far, Shahin, & Roushy, 2007). However, using physical methods, such as heat, high pressure, irradiation, sonication and electric pulses in food preservation often leads to the loss of nutrition in the food. The addition of synthetic fungicides is not appropriate for food because of their side effects on health and the environment as well as the development of fungicide-resistant fungi (Adebayo & Aderiye, 2011).

The use of naturally occurring antimicrobials in foods to prevent microbial spoilage and ensure food safety is in great demand. This is because this method has been shown to retain the nutritive value of food and to present no side effects for the consumer (Kumar, Mohandas, & Nambisan, 2013). The application of lactic acid bacteria (LAB) to inhibit mold growth might provide an interesting alternative to chemical fungicides and preservatives. In the past 10 years, the antifungal activity of LAB isolated from various sources has been reported. Yang and Chang (2008) isolated and identified antifungal *Lactobacillus plantarum* AF1 from kimchi. *Lactibacillus* spp., *Pediococcus* spp., *Enterococcus hirae* and *Weissella soli* with antifungal activity were isolated from various environmental samples including flowers, leaves, soil, honey, and chicken intestines (Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003). The

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prevalence and biodiversity of antifungal LAB in raw milk from cows, goats and ewes was reported and 94% of the antifungal isolates belonged to the genus *Lactobacillus* (Delavenne, Mounier, Déniel, Barbier, & Le Blay, 2012). Antifungal substances produced from LAB were reported to be organic acids, proteinaceous compounds and various low-molecular mass substances. These included phenyllactic acid; *p*-hydroxyphenyllactic acid; 3,6-bis(2-methylpropyl)-2,5-piperazinedion; cyclic dipeptides such as cyclo(Gly-L-Leu), cyclo(L-Phe-L-Pro) and cyclo (L-Phe-*trans*-4-OH-L-Pro); and certain organic acids such as acetic acid, propionic acid and lactic acid (Dal Bello et al., 2007; Magnusson et al., 2003; Ström, Sjögren, Broberg, & Schnürer, 2002; Yang & Chang, 2010).

Among antifungal LAB, *L. plantarum* was most frequently reported for strong antifungal activity with broad inhibitory spectrum over various fungal genera. Plus, multiple antifungal metabolites were detected and identified from this particular species. These included 3-phenyllactic acid from *L. plantarum* isolated from grass silage (Prema, Smila, Palavesam, & Immanuel, 2010), 3-hydroxylated fatty acids from *L. plantarum* MiLAB 14 isolated from lilac flowers (Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003), phenyllactic acid and 4-hydroxy-phenyllactic acid from *L. plantarum* 21B isolated from sourdough (Lavermicocca et al., 2000); benzoic acid, methylhydantoin, and mevalonolactone from *L. plantarum* VTTE-78076 isolated from beer (Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999) and several cyclic dipeptides from various strains of *L. plantarum* (Magnusson et al., 2003; Niku-Paavola et al., 1999; Ström et al., 2002).

This study aimed at evaluating the antifungal activity of *L. plantarum* K35 isolated from Thai fermented rice noodle (Kanom-jeen). The inhibition against growth of *A. flavus* and *A. parasiticus* and their production of aflatoxin were determined. The factors that influenced the inhibitory activity were investigated to enhance the sensitivity of GC–MS analysis for the assessment of the antifungal substances. The morphological alterations of the fungal mycelia exposed to *L. plantarum* K35 supernatant were also shown through SEM and TEM to determine the target sites of the antifungal compounds produced by *L. plantarum* K35.

2. Materials and methods

2.1. Media, microorganisms and cultivation conditions

A strong antifungal producing strain of *L. plantarum* K35, isolated from a fermented rice noodle, was cultivated in MRS broth (Himedia, Analyzed, India) at 37 °C for 24 h. The bacteria were stored as stock cultures in 30% glycerol at −196 °C under liquid nitrogen for one year.

Aflatoxin producing *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR) Phathumthani, Thailand. They were grown on Potato Dextrose Agar (PDA; Himedia, India) at 35 °C for 7 days.

2.2. Preparation of fungal spore suspension

The fungal spores were suspended in sterile 0.1% (v/v) Tween 80, and further diluted with Potato Dextrose Broth (PDB) to achieve a concentration of 10^6 spores/mL which was determined by using hemacytometer (BOECO, Germany). An appropriate dilution of fungal spore inoculum was dispensed either in the molten soft PDA (0.8% agar) or in the PDB containing microwells. This was done to achieve a final mold spore concentration of 10^5 spores/mL for the agar spot assay or broth microdilution assay, respectively.

2.3. Preparation of culture supernatant from L. plantarum K35

L. plantarum K35 was grown in MRS broth at 37 °C for 48 h. The bacterial cells were removed by centrifugation at 8500 rpm for 20 min at 4 °C to obtain a clear supernatant, which was then filter-sterilized through a sterile membrane with 0.22 μm pore size (Sartorius, Goettingen, Germany). The sterile supernatant was subjected to freeze-drying by freezing at −20 °C before the subsequent sublimation was performed at a capacity of 14 L/24 h using a Dura-Dry MP freeze-dryer (FTS System, USA). This was done for 15–18 h to yield the freeze-dried powder of the supernatant. The antifungal activity of the sterile liquid and the freeze-dried supernatant was quantitated and expressed as AU/mL and mg/mL, respectively.

2.4. Evaluation of antifungal activity of L. plantarum K35 by agar spot assay

L. plantarum K35 strain was tested for antifungal activity against the aflatoxin producing *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 by agar spot assay (Hassan & Bullerman, 2008). The assay consisted of two different agar layers, the bottom one was employed to support the growth of *L. plantarum* K35 and the top one for the fungi. *L. plantarum* K35 was cultivated in 10 mL of MRS broth at 37 °C for 24 h. A total of 5 μL of the culture broth was introduced as a single drop on the surface of MRS agar. The plate was then incubated at 37 °C for 48 h to allow the well-grown spot of *L. plantarum* K35 to form. The plate was then overlaid with 10 mL of soft PDA (0.8% agar) containing a final mold spore concentration of 10^5 spores/mL (2.2) before it was incubated at 35 °C for 48 h. The diameters of the inhibition zones around the LAB spots were measured using a vernier caliper. The evaluation was performed in triplicates over two separated runs.

2.5. Quantitative determination of antifungal activity of L. plantarum K35 against A. flavus and A. parasiticus

Antifungal activity was quantitated by broth microdilution assay according to the National Committee for Clinical Laboratory Standards or NCCLS (2002) and Yang and Chang (2010) with some modification. Microdilution tests were performed with a sterile 96-well microtiter plate (NUNC™, Denmark). The freeze-dried powder was dissolved in 50 mM phosphate buffer (pH 7.0) to obtain an initial concentration of 19.5 mg/mL. The sterile liquid or freeze-dried supernatant was dispensed into each well (180 μL) in a two-fold dilution manner using PDB. Twenty microliters of fungal spore suspension were prepared according to the method above (see 2.2) and added to 180 μL PDB to achieve a final concentration of 10^5 spores/mL in each well. The microtiter plate was then incubated at 35 °C, and fungal growth was determined visually after 48 h of incubation. The highest dilution (A) at which fungi inhibition was observed (optically clear or absence of growth was compared with a control well without fungal inoculum). The antifungal activity of the sterile liquid supernatant was expressed in arbitrary units (AU/mL), which were calculated by (1000/180) × A. That of the freeze-dried one was reported as the MIC (minimum inhibitory concentration) value defined by NCCLS (2002). The antifungal activity of each supernatant was evaluated in triplicates over two separate runs.

The fungal viability of the dilution level which showed no visible growth was also determined by transferring and spreading 10 μL of the aliquot on PDA agar plate before incubation at 35 °C for 48 h. The highest dilution required to completely destroy the test fungi (no growth on the agar plate) was defined as minimum fungicidal concentration (MFC).

2.6. Factors influencing antifungal activity of L. plantarum K35

2.6.1. Incubation period

L. plantarum K35 was cultivated in MRS broth at 37 °C. After 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h of incubation, its growth and antifungal activity were determined. The clear supernatants were filter-sterilized through a sterile membrane with 0.22 μm pore size. The inhibition activity of the supernatants was determined by the broth microdilution method mentioned above in 2.5. The optimum incubation period showing the highest inhibitory activity was applied for LAB cultivation in the further experiment.

2.6.2. Incubation temperature

L. plantarum K35 was grown in MRS broth at 25, 37 and 45 °C. The antifungal activity of the supernatant was determined by the broth microdilution method mentioned above in 2.5.

2.6.3. Initial pH

L. plantarum K35 was grown in MRS broth with a variation of the initial pH of 5.0, 5.5. 6.0, 6.5, 7.0, 7.5 and 8.0 at 37 °C. The antifungal activity of the supernatant was determined by the broth microdilution method mentioned above in 2.5.

2.7. Characterization of the antifungal compounds produced by L. plantarum K35

The antifungal substances produced by *L. plantarum* K35 were characterized based on their sensitivity towards changes of pH, heat and protease enzymes. The freeze-dried powder of the sterile supernatant was dissolved in 50 mM phosphate buffer (pH 7.0) for determining the antifungal activity (Coloretti et al., 2007). For the pH sensitivity, the supernatant was adjusted to pH 4, 5, 6 and 7 with 1 M HCl and 1 M NaOH before the inhibitory activity against the aflatoxin producing *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 was assayed. The sterile MRS broth adjusted to pH 4, 5, 6 and 7 was applied in the control well.

The sensitivity to heat was evaluated by subjecting the supernatant to a number of thermal treatments: 63 °C for 30 min; 100 °C for 30 min; and 121 °C for 15 min. The antifungal activity was then determined using the broth microdilution assay (2.5).

The sensitivity to various proteolytic enzymes was evaluated with the neutralized freeze-dried supernatant (pH 7.0) and the addition of 50 U/mL catalase (Sigma–Aldrion Chemie, Steiheim, Germany) to rule out the inhibition of acid and hydrogen peroxide. The sensitivity to proteolytic enzymes was investigated by the addition of proteinase-K and trypsin (Sigma–Aldrion Chemie, Steiheim, Germany) at a final concentration of 1 mg/mL. The samples were incubated for 3 h at 37 °C. The reaction was then stopped by boiling for 5 min, and the antifungal activity was determined by the broth microdilution method mentioned in 2.5.

2.8. Effects of the culture supernatant from L. plantarum K35 on growth and aflatoxin production of A. flavus and A. parasiticus in liquid medium

The fungal survival was evaluated after the exposure to LAB supernatant. The serially diluted supernatant in PDB (45 mL) was dispensed into 125 mL conical flasks. These were inoculated with 5 mL of spore suspension from 2.2 to achieve final concentration of 10^5 spores/mL and incubated at 35 °C. The survival of mold growth at 0, 3, 6, 9, 12, 15, 18, 21, 24, 30 and 48 h of incubation was determined by spreading the appropriate dilutions on PDA plates. The mold colonies were counted and expressed as log CFU/mL.

The tests for the inhibition of fungal growth and aflatoxin production were assayed in flask cultures. Semi-synthetic yeast extract-sucrose (YES) containing 2 g yeast extract and 15 g sucrose in 100 mL of distilled water was used for this study (Vergopoulou, Galanopoulu, & Markaki, 2001). Five milliliters of spore suspension (in sterile 0.1% Tween 80) were prepared from *A. flavus* and *A. parasiticus* cultivated in YES agar at 35 °C for 7 days and diluted to achieve 10^6 spores/mL. The diluted suspension (5 mL) was transferred to 45 mL of YES broth (with the addition of 0, 4.40, 5.87, 8.80 and 17.6 mg/mL of the freeze-dried supernatant). Each flask was incubated at 35 °C under a shaking speed of 200 rpm. After 7 days of incubation, the fungal dry weight and aflatoxin production were determined (Tolouee et al., 2010).

The aflatoxin content in the cultured supernatant was determined with a DOA-aflatoxin ELISA test kit (Department of Agriculture, Thailand). A direct competitive ELISA was employed for this measurement, and the analysis procedure was performed according to the manufacturer's instruction (Rammanee & Hongpattarakere, 2011). The culture broth was filtered through filter paper (Whatman No.1). The fungal residue was washed with sterile water, and then allowed to dry at 80 °C until a constant weight was obtained. The aflatoxin content in the culture supernatant was determined by adding 100 mL of 70% methanol to 20 mL supernatant. The supernatant–methanol mixture was then shaken at 300 rpm for 30 min, and then filtered through filter paper. The aliquot obtained was further analyzed for aflatoxin as described in detail by Rammanee and Hongpattarakere (2011).

2.9. Chemical composition analysis of the culture supernatant from L. plantarum K35

2.9.1. Ethylation

The level of phenyllactic acid analysis was performed as previously described by Ryan, Bello, Czerny, Koehler, and Arendt (2009). Initially, 1 mL of supernatant was subjected to esterification to yield ethyl ester derivatives by mixing with 10 mL of absolute ethanol and 15 drops of sulfuric acid (97% v/v). The ethylation reaction was conducted at 80 °C with continuous stirring at 200 rpm for 1 h. After cooling, 20 mL of water were added. Each sample was extracted 5 times with 50 mL of dichloromethane. Sodium sulfate (50 g) was added and the mixture was stirred at 200 rpm for 10 min and then filtered through a 0.45 μm membrane filter. The dichloromethane phase was then collected and pooled. After the removal of the solvent using a vacuum evaporator operated at 50 °C, the residue was dissolved in 5 mL of dichloromethane and subjected to Gas Chromatography–Mass Spectrophotometry (GC–MS) analysis.

2.9.2. GC–MS analysis

The analysis of the composition of the supernatant was performed using GC–MS on the Thermo Scientific TRACE GC Ultra Gas Chromatograph coupled to the ISQ Single Quadrupole Mass Spectrometer (Thermo scientific Inc, USA) with a TR-WaxMS column (length 30 m, film thickness 0.25 μm, ID 0.25 mm). An inlet temperature of 250 °C with the split ratio 10:1 was employed. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The esterified sample prepared in 2.9.1 was applied and maintained at 40 °C for 2 min. The temperature was then increased at a rate of 6 °C/min to 230 °C, at which the temperature was 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, scan mass range of 35–500 amu, and MS transfer line at temperature of 245 °C with 5 min solvent delay time. The components were identified based on a comparison of their relative retention times and fragmentation patterns of mass spectra compared to those reported in the literature as well as the Wiley, 275.L data library of the GC/MS system.

2.10. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Fungal materials obtained from 7-day-old cultures grown on PDB with or without the supernatant were processed for SEM and TEM. The SEM was performed on fungal materials according to Tolouee et al. (2010). Fungal mycelia were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 3 h. The fixed mycelia were washed with the same buffer three times each for 10 min, and then post-fixed with 1% osmium tetroxide (OsO_4) in the same buffer at room temperature for 2 h. The specimens were dehydrated through a graded series of ethanol (50%, 70% 80% and 90%) for 10 min. The dehydrated specimens were dried in a critical point drier (CPD) with liquid carbon dioxide, and sputter-coated with gold. Observations were carried out with a scanning electron microscope (Qunta 400; FEI Ltd, UK).

For the TEM, the analysis was performed according to Rammanee and Hongpattarakere (2011). Fungal mycelia were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at room temperature. After washing three times in 0.1 M phosphate buffer (5 min each time), the specimens were post-fixed in 1% osmium tetroxide (OsO_4) as for the SEM. The samples were dehydrated in a graded series of ethanol (30%, 50%, 70%, 80% and 100%, twice for 5 min each). The fixed mycelia were processed in graded propylene oxide twice (15 min each), in propylene oxide/Epon 812 (1:1) for 1–2 h, and were finally embedded in 100% Epon 812. The polymerization undertaken to form a specimen block was achieved at 70–80 °C overnight. The specimen blocks were sectioned using an ultramicrotome (Drukker International, Netherland) with 80–90 nm thickness (golden yellow section). The section was placed on 200-mesh copper grids and stained with 5% uranyl acetate (dissolved in 70% methanol) for 15 min. The stained sections were examined with a transmission electron microscope (JEM-2010; JEOL Ltd.,Tokyo, Japan).

3. Results and discussion

3.1. Inhibition of L. plantarum K35 on growth and aflatoxin production of A. flavus and A. parasiticus

The strong antifungal activity of *L. plantarum K35* against the aflatoxin producing *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 is shown in Fig. 1. Its antifugal levels measured by broth dilution assay against *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 were 33.33 AU/mL and 27.77 AU/mL, respectively. In relation to this, several researchers have reported that *L. plantarum* has the ability to inhibit a broad spectrum of fungi (Magnusson et al., 2003; Sjögren et al., 2003; Ström et al., 2002). Ström et al. (2002) reported that *Aspergillus fumigatus*, *Aspergillus nidulans, Penicillium commune* and *Fusarium sporotrichioides* were inhibited by *L. plantarum* MiLAB393. Similarly, *L. plantarum* AF1 showed broad inhibition against *Aspergillus, Penicillium, Epicoccum* and *Cladosporium* (Yang & Chang, 2010). Many strains of *L. plantarum* isolated from flowers inhibited *A. fumigatus* J9, *A.nidulans* J283 (FSGC A4 wt), *P. roqueforti* J268 (IBT 6754), *P. commune* J238 (IBT 12400), and *F. sporotrichioides* J304 (ITEM168) (Magnusson et al., 2003).

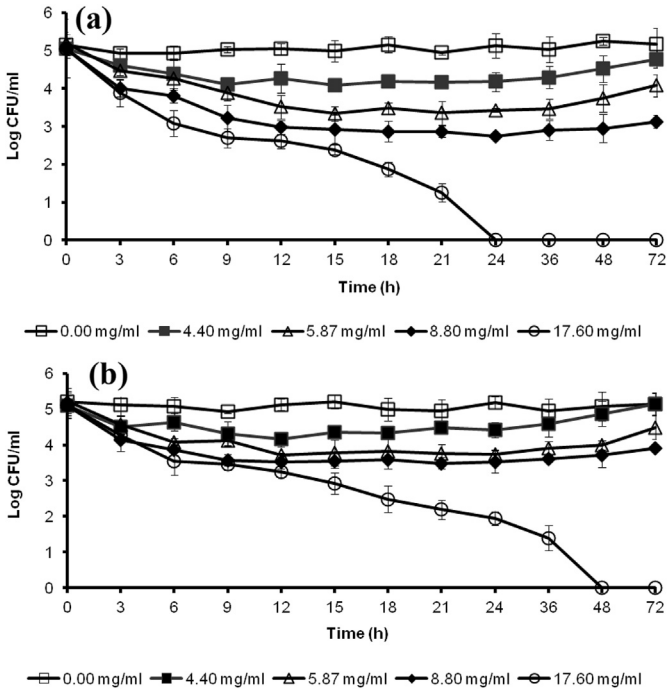


Fig. 2. Antifungal activity of supernatant from *L. plantarum* K35 at the concentration of 0, 4.40, 5.87, 8.80 and 17.6 mg/mL on growths of (a) *A. flavus* TISTR 3041 and (b) *A. parasiticus* TISTR 3276 tested in potato dextrose broth at 35 °C for 72 h. Data spots were mean values of triplicate determinations ± standard deviation.

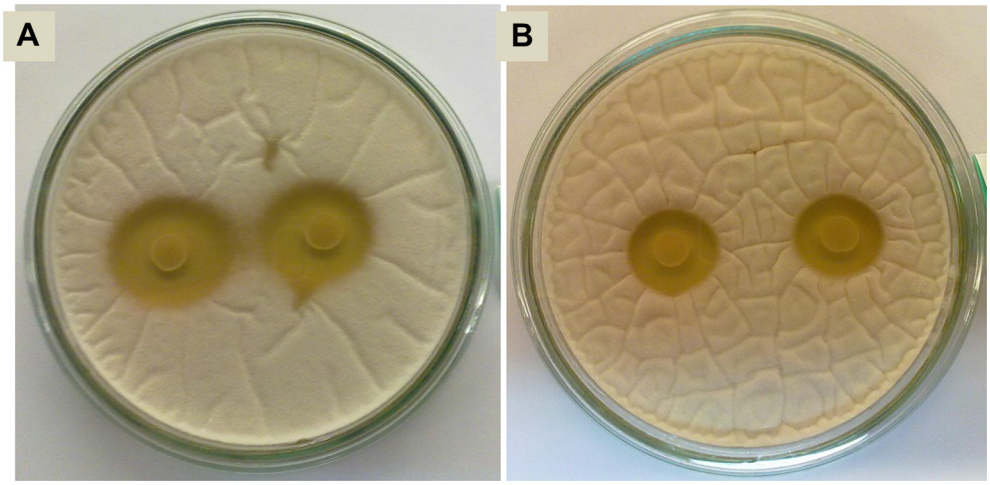


Fig. 1. Inhibitory zone formed around *L. plantarum* K35 against (A) *A. parasiticus* TISTR 3276 and (B) *A. flavus* TISTR 3041 after 2 days of incubation.

Table 1
Inhibitory effect of supernatant from *Lb. plantarum* K35 on growth and aflatoxin production of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 in YES medium.

Supernatant concentration (mg/mL)	*A. flavus* TISTR 3041		*A. parasiticus* TISTR 3276	
	Mycelium dry weight (mg/mL)	AFB1 concentration (μg/mL)	Mycelium dry weight (mg/mL)	AFB1 concentration (μg/mL)
0.00	18.67 ± 4.51^{b}	5.81 ± 0.50^{d}	18.83 ± 2.47^{c}	5.08 ± 0.55^{d}
4.40	15.33 ± 3.06^{b}	5.38 ± 1.14^{d}	18.83 ± 1.61^{c}	4.62 ± 0.84^{d}
5.87	12.67 ± 2.89^{b}	3.42 ± 0.27^{c}	15.00 ± 3.46^{c}	3.78 ± 0.34^{c}
8.80	6.00 ± 4.58^{a}	1.82 ± 0.52^{b}	8.07 ± 4.56^{b}	1.93 ± 0.75^{b}
17.6	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Data were mean values of five replicate determinations ± standard deviation. Means within columns not sharing the same superscript are significantly different ($p < 0.05$).

The supernatant of *L. plantarum* K35 significantly reduced the growth and production of aflatoxin of *A. flavus* and *A. parasiticus*. At concentrations of 5.87, 8.80 and 17.6 mg/mL *A. flavus* was more sensitive than *A. parasiticus* with 30.29, 40.93, 48.17% and 27.51, 31.23 36.77% inhibition, respectively after 12 h exposure. Complete inhibition was observed at concentrations of supernatant of 17.6 mg/mL after 24 h and 48 h exposure, respectively (Fig. 2a and b). The effects of the supernatant from *L. plantarum* K35 on aflatoxin production and dry biomass in the Yeast Extract Sucrose medium are shown in Table 1. The significant reduction of the fungal growth and aflatoxin production was associated with the increase of the supernatant concentration. At 5.87 mg/mL of the supernatant, the aflatoxin production of *A. flavus* and *A. parasiticus* was significantly ($p < 0.05$) reduced from 5.81 ± 0.50 and 5.08 ± 0.55 μg/mL to 3.42 ± 0.27 and 3.78 ± 0.34 μg/mL, respectively. The similar inhibition of aflatoxin production by *Lactobacillus* cell-free supernatant was reported (Karunaratne, Wezenberg, & Bullerman, 1990). Coallier-Ascah and Idziak (1985) reported a significant inhibition of aflatoxin production by *Lactobacillus*-cell free supernatants. This author also suggested that the inhibition was related to the heat stable components with low-molecular weight.

3.2. Factors influencing antifungal activity of L. plantarum K35

Antifungal substances produced by *L. plantarum* K35 were optimized to facilitate the chemical analysis. As previously reported, the incubation period, growth temperature and initial pH are critical factors that significantly influence LAB growth and the amount of antifungal metabolites produced (Batish, Roy, Lal, & Grover, 1997; Dalié, Deschamps, & Richard-Forget, 2010). The production of antifungal metabolites by *L. plantarum* K35 was initiated at the log phase (6–12 h) of growth in MRS broth. The highest activity was shown when LAB growth reached the stationary phase at 48 h and remained stable thereafter (Fig. 3a). The antifungal compounds produced by *L. plantarum* K35 reached the highest level at 37 °C of incubation in MRS broth with an initial pH of 6.5 (Fig. 3b, c). But there was no significance with the cultivation at pH 6.0 and 6.8. However, lower activity was observed at 25 °C and 45 °C ($p < 0.05$). The results indicated that the production of antifungal substantces was growth-associated. This observation was in accord with previous studies carried out by Sathe, Nawani, Dhakephalkar, and Kapadnis (2007) and Magnusson and Schnürer (2001). However, Reddy and Ranganathan (1985), cited by Dalié et al., 2010) reported that the greatest production of antifungal metabolites by *Lactococcus lactis* subsp. *diacetylactis* occurred after three to four days of incubation at 25 °C. The antifungal activity of *L. plantarum* CUK501 reached the maximal level at the end of its logarithmic phase and the maximum yield of antifungal metabolites was obtained at 30 °C (Sathe et al., 2007). The activity declined over 48 h of incubation, when the cell growth entered the stationary phase. Magnusson and Schnürer (2001) reported that the antifungal activity of *Lactobacillus coryniformis* subsp. *coryniformis* Strain Si3 was at its maximum after 48 h of incubation. These contradictory results indicate that the conditions

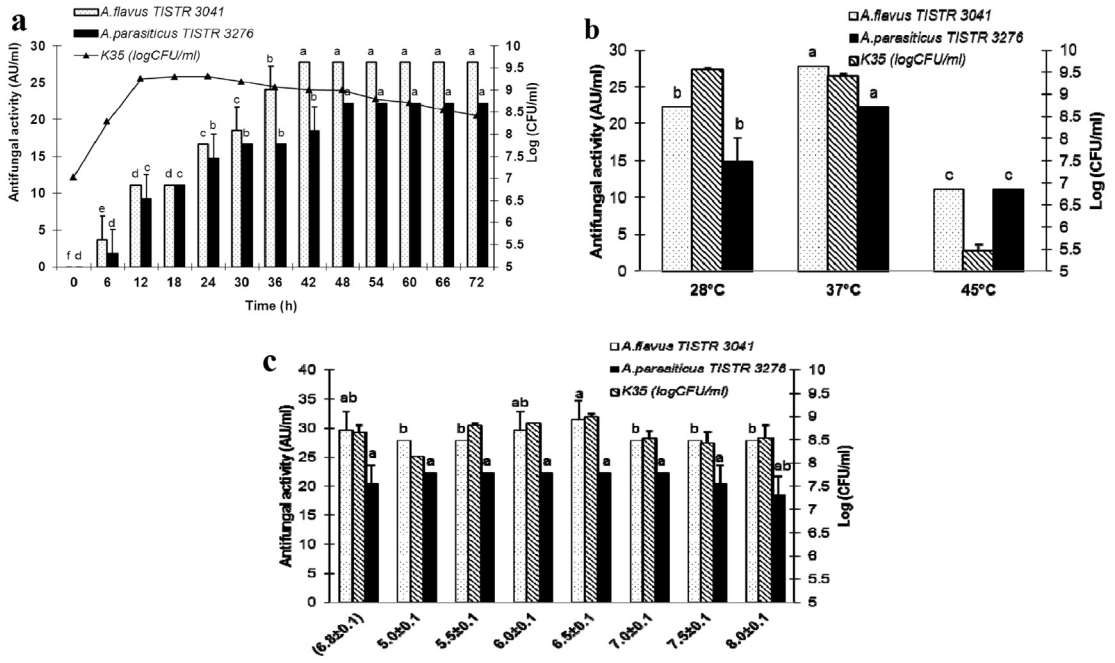


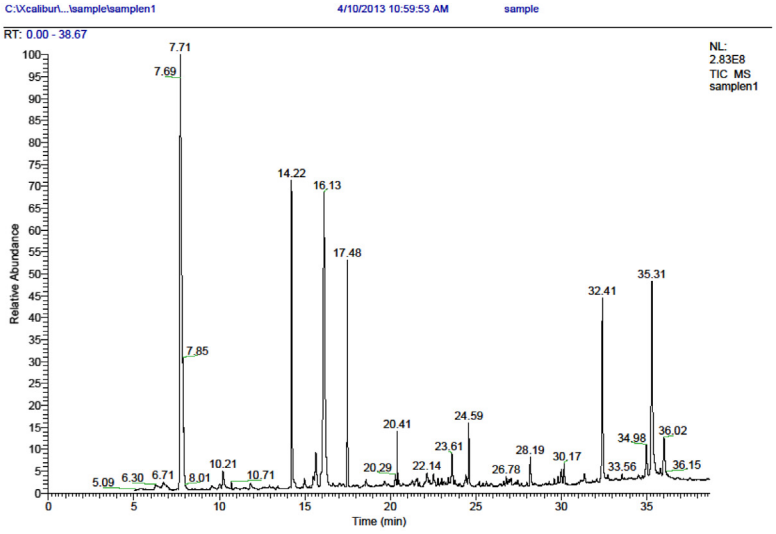
Fig. 3. Effects of incubation time (a), temperature (b), and initial pH (c) on growth of *L. plantarum* K35 and its antifungal activity against *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276. Data were mean values of triplicate determinations ± standard deviation. Values followed by different letters were significantly different ($p < 0.05$).

required for the production of LAB antifungal metabolites are not only genus/species specific but also strain dependent.

3.3. Characteristics and composition of the antifungal compounds produced by L. plantarum K35

When *L. plantarum* K35 supernatant was neutralized from pH 4.0 to 7.0, the inhibitory activity against *A. flavus* and *A. parasiticus* decreased from MIC values of 11 and 22 to 88 mg/mL and 176 mg/mL, respectively (data not shown). This pH-dependent antifungal activity was similar to many previous reports (De Muynck et al., 2004; Niku-Paavola et al., 1999; Yang & Chang, 2008). The antifungal substances of *L. plantarum* K35 remained active even though the extreme heat treatment of 121 °C 15 min was applied (data not shown). Similarly, the activity remained stable after the treatments of catalase and various proteolytic enzymes including trypsin and proteinase-K. Therefore, no hydrogen peroxide or proteinaceous compounds contributed to the antifungal activity of *L. plantarum* K35. The pH-dependent characteristic was consistent with the antifungal substances of the cell-free supernatant from *L. plantarum* AF1 (Yang & Chang, 2008) and *L. plantarum* VTT E-78076 (Niku-Paavola et al., 1999). The activity of *L. plantarum* AF1 was stable at pH values between 3.0 and 4.0, but rapidly decreased over pH 5.0 (Yang & Chang, 2008).

The identification of 10 compounds from a total of 22 components (Fig. 4) was revealed from the chemical composition analysis of *L. plantarum* K35 supernatant using GC–MS. Interestingly, *L. plantarum* K35 produced multiple antifungal compounds with major constituents of lactic acid (33.75%), 2-butyl-4-hexyloctahydro-1H-indene (19.55%), 9-octadecenoic acid or oleic acid (10.52%), palmitic acid (7.27%), linoleic acid (2.11%), stearic acid (1.55%), 3-phenyllactic acid (1.42%) and pyroglutamic acid or 5-oxo-2-pyrrolidine-carboxylic acid (1.07%). Unfortunately two abundant compounds at retention times of 14.22 (7.35%) and 17.48 min (4.32%) could not be identified. Apart from lactic acid, the 2-butyl-4-hexyloctahydro-1H-indene was, for the first time, reported as the second abundance in *L. plantarum*. This compound was identified in the broadest spectrum antimicrobial fraction from a sponge associated microbe (Dash, Jin, Lee, Xu, & Qian, 2009). Long chain fatty acids, particularly C_{16} and myriad of saturated and unsaturated C_{18},



Retention time	Composition	% Relative concentration	Molecular weight
7.71	Lactic acid ($C_3H_6O_3$)	33.75	90.08
14.22	Unidentified	7.35	-
16.13	1H-Indene,2-butyl-4-hexyloctahydro ($C_{19}H_{36}$)	19.55	264
17.48	Unidentified	4.32	-
20.41	Unidentified	0.85	-
23.61	3-Phenyllactic acid ($C_{11}H_{14}O_3$)	1.42	194
24.59	2,4-Di-tert-butylphenol ($C_{14}H_{22}O$)	1.84	206
28.19	Pyroglutamic acid ($C_5H_7NO_3$)	1.07	129.11
30.17	5-Methyl-1-phenylbicyclo (3,2,0) heptanes ($C_{14}H_{18}$)	0.81	186.29
32.41	Palmitic acid ($C_{16}H_{32}O_2$)	7.27	256
34.98	Stearic acid ($C_{18}H_{36}O_2$)	1.55	284
35.31	Oleic acid ($C_{18}H_{34}O_2$)	10.52	282
36.02	Linoleic acid ($C_{18}H_{32}O_2$)	2.11	280

Fig. 4. Gas chromatogram and chemical composition of *L. plantarum* K35 supernatant.

were also secreted by *L. plantarum* K35. Oleic acid was found in a relative amounts as the third abundant antifungal component produced by *L. plantarum* K35. Walters, Raynor, Mitchell, Walker, and Walker (2004) previously reported the inhibitory activities of linolenic acid, linoleic acid, and oleic acid on the growth of plant pathogenic fungi including *Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avenae* and *Crinipellis perniciosa*.

The identified antifungal compounds produced by *L. plantarum* K35 shared common features such as being small molecules with the molecular masses ranging from 90 of lactic acid to 284 of stearic acid (Fig. 4). Apart from the lactic acid and fatty acids, these compounds also shared aromatic or heterocyclic structures with hydrophobic characteristics. A cyclic compound, pyroglutamic acid, was introduced as an antimicrobial agent produced by *Lactobacillus casei* ssp. *casei* LC-10 and *L. casei* ssp. *pseudoplantarum* LB1931 (Huttunen, Noro, & Yang, 1995). Surprisingly, 3-phenyllactic acid (PLA), a well-known LAB antifungal compound, was produced by *L. plantarum* K35 at a low relative concentration. PLA has been considered as an antifungal compound marker produced from various LAB. It was assumed that LAB antifungal activity was positively related to the metabolic content of PLA (Lavermicocca et al., 2000; Schnürer & Magnusson, 2005; Wang et al., 2012). PLA produced by *L. plantarum* 21B was shown to possess efficient antifungal activity against *Penicillium corylophilum*, *Penicillium roqueforti*, *Penicillium expansum*, *Aspergillus niger*, *A. flavus* and *F. graminearum* (Lavermicocca et al., 2000, Lavermicocca, Valerio, & Visconti, 2003). The identified antifungal compounds from *L. plantarum* K35 supported what many researchers have concluded that LAB antifungal substances had a low-molecular-weight (Dalié et al., 2010; Magnusson et al., 2003; Niku-Paavola et al., 1999). The presence of multiple antifungal components may explain the broad spectrum of inhibition of *L. plantarum* K35 against many foodborne pathogens and other fungi including *Aspergillus fumigatus*, *A. niger* and *Penicillium* sp. (data not shown).

The antifungal compounds from *L. plantarum* K35 were different from those previously mentioned in the research reports. The active compound from *L. plantarum* AF1 was identified as $C_{12}H_{22}N_2O_2$, 3,6-bis(2-methylpropyl)-2,5-piperazinedion with a molecular mass of 226 (Yang & Chang, 2010). Ström et al. (2002) identified cyclo (L-phe-L-pro), cyclo (L-phe-*trans*-4-OH-L-pro) and phenyllactic acid as antifungal substances produced by *L. plantarum* MiLAB 393. Furthermore, Niku-Paavola et al. (1999) demonstrated that, *L. plantarum* synthesized a number of antifungal substances, including benzoic acid, 5-methyl-2,4-imidazolidinedione (methylhydantoine), tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one (mevalonolactone) and cyclo (glycyl-L-leucyl). However, lactic acid did not solely contribute to such activity. These studies indicate that the antifungal compounds are diversified and widely

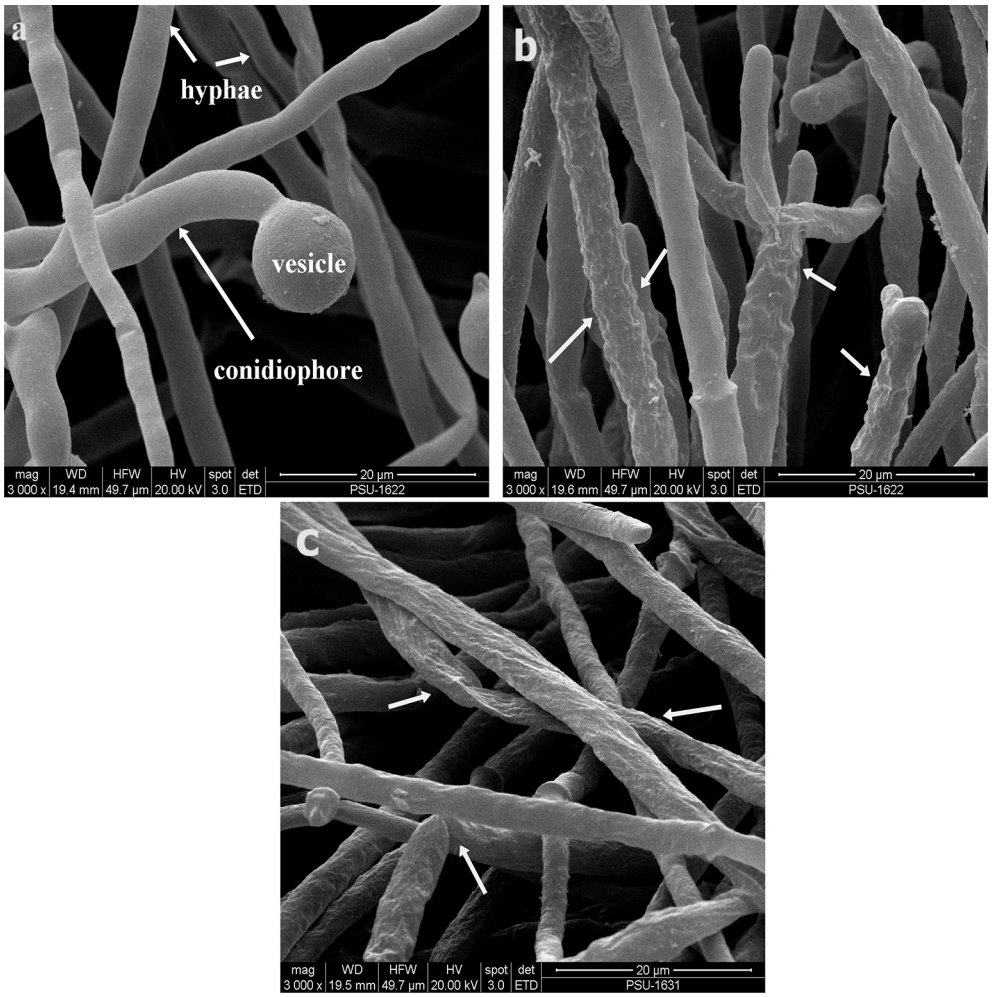


Fig. 5. Scanning electron micrographs (SEM) of 7-day-old *A. parasiticus* TISTR 3276 (a, c) and *A. flavus* TISTR 3041 (b) with or without exposure to 5.87 mg/mL supernatant from *L. plantarum* K35. Control *A. parasiticus* TISTR 3276 (a) appear as normal structures of tubular-shape hyphae, conidiophores and vesicle with smooth surface. The supernatant treated mycelia (b and c) are markedly shriveled and crinkled (white arrow).

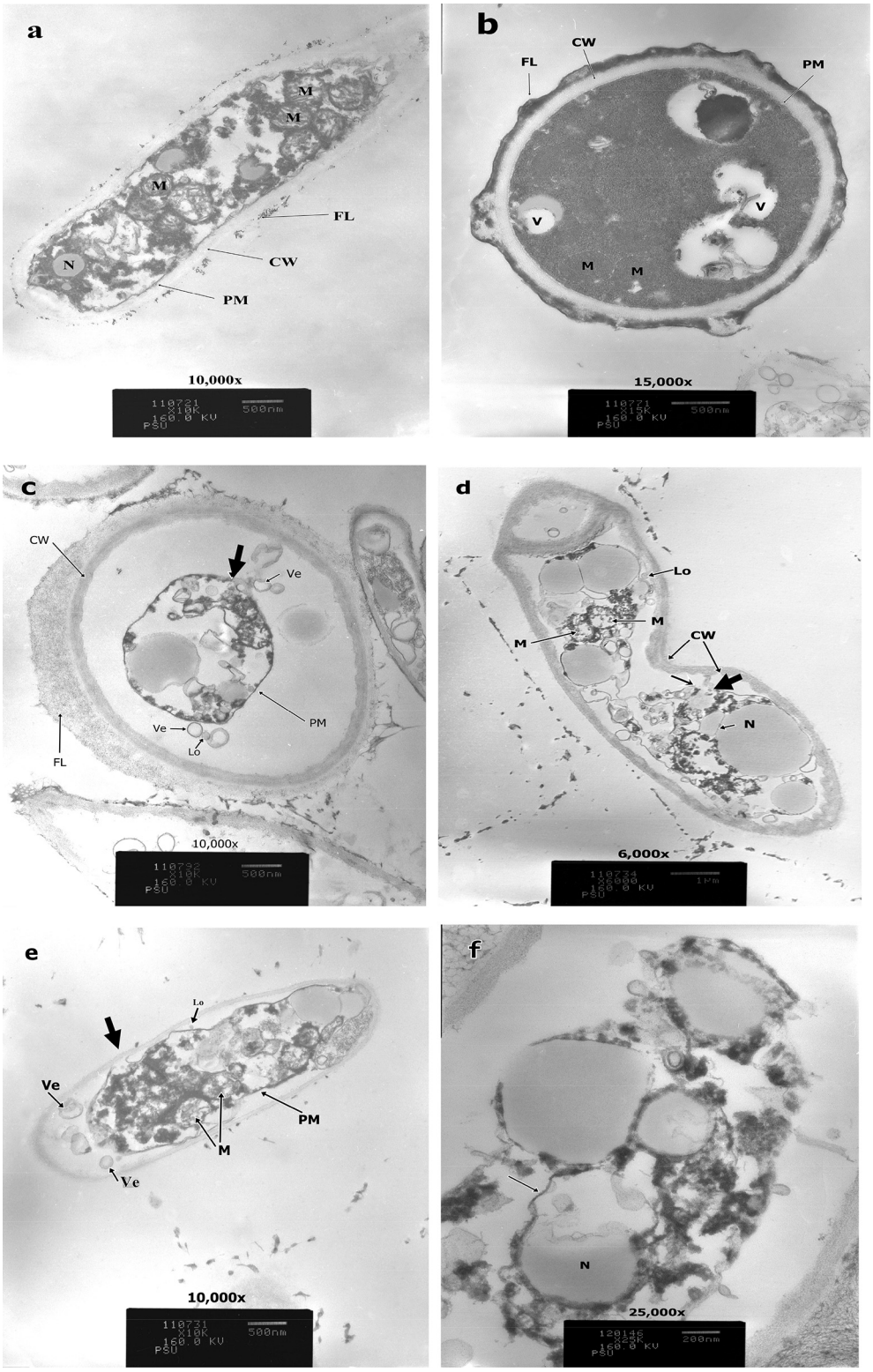


Fig. 6. Transmission electron micrographs (TEM) of 7-day-old *A. parasiticus* TISTR 3276 (a, c, e) and *A. flavus* TISTR 3041 (b, d, f) with or without exposure to 5.87 mg/mL supernatant from *L. plantarum* K35. Non-treated *A. parasiticus* TISTR 3276 (a) and *A. flavus* TISTR 3041 (b) with normal fibrillar layer (FL), cell wall (CW), plasma membrane (PM), nucleus (N), vacuole (V), and mitochondria (M). The supernatant-treated mycelia show thicken fibrillar layer (c), thinning (c, e) and collapsed cell wall with rough, irregular inner surface (d), complete detachment and disruption of plasma membranes (thick arrow in c, d and e) leading to loss of cytoplasmic content with the formation of small lomasomes (Lo) and membrane-bound vesicles (Ve). Disruption and degeneration of various fungal organelles, such as destruction of nucleus membrane (arrow in f), loss of mitochondrial cristae (arrow in d and e) and nucleus fusion (arrow in d) were clearly illustrated.

distributed among different strains of *L. plantarum*. At pH 4.0 and above, lactic and 3-phenyllactic acids may not be considered to play a major contribution to the antifungal activity of *L. plantarum* K35. According to theories about weak-acids, only hydrophobic undissociated molecules of organic acids can penetrate microbial cell membrane leading to a progressive decrease of intracellular pH and the collapse of proton motive force (Schillinger & Villarreal, 2010). Since the pKa of lactic acid and 3-phenyllactic acid are 3.8, and 3.5, respectively; the adjustment of the LAB supernatant to a pH above 4.0 can remove their inhibitory activity. Our results showed that multiple compounds may significantly contribute and in a synergistic sense to the fungal inhibition of *L. plantarum* K35 apart from lactic acid and 3-phenyllactic acid.

3.4. Fungal ultrastucture alterations through SEM and TEM observations

The morphological changes in 7-day-old *A. flavus* and *A. parasiticus* exposed to the supernatant from *L. plantarum* K35 at the MIC concentration were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM observation showed that untreated *A. flavus* and *A. parasiticus* hyphae retained complete tubular shapes with the normal appearance of a barrel-like formation and smooth cell walls (Fig. 5a). After being exposed to 5.87 mg/mL of the supernatant from *L. plantarum* K35, the aberrant and distorted morphologies of the fungal hyphae were distinctively observed. Markedly shriveled, and crinkled cell walls and flattened hyphae were evident from the SEM (Fig. 5b, c).

The many destructive sites of fungi revealed by TEM supported that multiple compounds contributed to the antifungal activity of *L. plantarum* K35. The TEM observations clearly showed that the cell walls of the untreated fungi are uniform and protected thoroughly by an intact fibrillar layer. The plasma membrane was unfolded with a uniform shape. The septum and all organelles including mitochondria, vacuole and nucleus had normal appearances (Fig. 6a, b). After being exposed to 5.87 mg/mL of supernatant from *L. plantarum* K35, the plasma membrane seemed to be the primary target. The complete detachment from the cell wall and lysis or disruption and disappearance of the plasma membranes were obvious, leading to a marked depletion of the cytoplasm content with the formation of small lomasomes and membrane-bound vesicles. The disruption and degeneration of various fungal organelles, such as the loss of mitochodrial cristae and fusion of the nucleus are clearly illustrated in Fig. 6c to f. The diminution and disorganization of the plasma membrane as well as the disintegration and disruption of the membranous organelles seemed to be responsible for cell death. The SEM and TEM showed that supernatant from *L. plantarum* K35 targeted multiple sites of aflatoxin producing *A. flavus* and *A. parasiticus*, particularly the lipid bilayer and cell wall.

The TEM observation was correlated to the chemical analysis showing the presence of multiple antimicrobial components. Various fatty acids are well-known as membrane targeting antimicrobials. Many mechanisms were involved in the membrane disorganization of fatty acids. Palmitic acid was inserted into the lipid bilayer through ATP-induced sequestration of calcium causing a reduction of the phospholipid content (Herbette, Favreau, Segalman, Napolitano, & Watras, 1984). In addition, various antimicrobial mechanisms of unsaturated C_{18} were noted. Oleic acid was well-characterized for its ability to incorporate and alter the lipid bilayer causing changes in membrane permeability and leading to disruption of the membrane (Herbette et al., 1984). Free fatty acids were antimicrobials targeting not only the cell membrane (Avis, 2007) but also specific enzymes and metabolic pathways, paricularly those involved in the fatty acid synthesis (Zheng et al., 2005). Linoleic, oleic and other long chain unsaturated fatty acids were proven to inhibit bacterial enoyl-acyl carrier protein reductase, an essential enzyme for bacterial fatty acid synthesis (Zheng et al., 2005).

Interestingly, a thicken fibrilla layer (Fig. 6c), and a thinner collasped cell wall with a rough, irregular inner surface (Fig. 6d) were also clearly observed. Although the cell wall lost its integrity, it still remained intact. This observation indicated the presence of other inhibitiory compounds targeting the cell wall apart from those attacking the lipid bilayer. The TEM data therefore supported the composite analysis that multiple components may function in concert to effectively inhibit the aflatoxin producing fungi by attacking multiple sites of fungal hyphae.

4. Conclusion

L. plantarum K35 displayed the potent antifungicidal activity against aflatoxin producing strains of *A. flavus* and *A. parasiticus*. Many antifungal components were analyzed and identified from the supernatant of *L. plantarum* K35. The irreversible damage and morphological alteration were clearly evident in various membrane-bound structures and cell wall of the fungi. Therefore, the presence of many antifungal substances may be responsible for the massive destruction by attacking multiple targets. Further application of LAB antifungal compounds may provide a promising alternative to chemical preservatives. The mixture of these potential biopreservatives could effectively prevent fungal spoilage and aflatoxin formation in food and animal feed.

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