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Anti - *Aspergillus flavus* Activity of *Lactobacillus plantarum* and *Pediococcus acidilactici* Isolated from Breast Milk

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ABSTRACT

The breast milk is not only the best and optimal natural source of essential nutrients for babies, but also provides a broad range of bioactive compounds, as well as its own microbiota, therefore helps to protect body against many diseases too. Lactic acid bacteria (LAB) are the major group of the breast milk microbiota and acts as biocontrol agent in milk. In this study LAB were isolated from the breast milk. Their antifungal activity against aflatoxin producing Aspergillus flavus was investigated with four methods: Study of the LAB isolates influence on biomass production of A. flavus in MRS medium, determination of their inhibitory activity against A. flavus on PDA medium, dual culture overlay assay and finally, effect of LAB cell-free supernatants (CFS) against mycelia growth. The CFS also was analyzed by HPLC and titration experiments. The results indicated that seven isolated LAB had good inhibitory activity against A. flavus growth. Four isolates of LAB were identified as Pediococcus acidilactici and three of them were identified as Lactobacillus plantarum by 16s rDNA sequencing assay. The cell-free supernatants of L. plantarum RS2 expressed strong anti A. flavus activity. Analyzing of their CFS showed that, this strain had significant amounts of propionic acid and hydrogen peroxide in the CFS that might explain the high antifungal activity.

Keywords: *Lactobacillus plantarum*; *Pediococcus acidilactici*; Biocontrol; Breast milk; *Aspergillus flavus*

INTRODUCTION

The breast milk is not only the best and perfect natural nutrient for babies, but also helps to protect body against many diseases. The breast milk feeding ensures optimum nutrition for rapid infants development [1,2]. The breast milk microbiota characterization has showed that it contains a limited range of gram-positive bacteria [3]. Between them, lactic acid bacteria (LAB) are often detected in this complicated biological species-specific fluid. LAB are generally recognized as safe (GRAS) and in addition to their local activities as commensal bacteria, they look like to do an important play on many host activities, as well as the protection against pathogens or the modulating maturation of the neonates immune system [4,5].

Recently, the bio-preservation as the use of the safe microorganisms and/or their metabolites to inhibit spoilage and to increase shelf life of food and consumer' demands, has been considered by researchers and producers. The application of LAB as bio-preservation microorganisms has a growing tendency in food preservation for their antagonistic ability against several food-borne pathogens. Their preventing effect against some bacteria and fungi is through some mechanisms including production of organic acids, hydrogen peroxide, fatty acids and bacteriocins, reducing environmental pH and stimulation of the immune response [6–10]. LAB are a widespread group of gram positive, catalase negative, usually non-motile, non-sporing rods and cocci that fermenting carbohydrates and producing lactic acid as the main end product [11]. The antifungal strains of LAB have been isolated from various environments [12].

Fungal food spoilage is one of the major origins of food hazards and economic damages overall the world [13]. The mycotoxigenic molds like *Aspergillus flavus* are the major food safety concerns and have a very important role in food spoilage and decreasing the quality of food offered in the market. *A. flavus* is one of the main aflatoxin producer [14–16]. Aflatoxin is among the most toxic substances known yet [17]. In public health, aflatoxin is a worldwide serious problem. Aflatoxin is toxic to animals and human beings, and has mutagenic and teratogenic effects and can induce tumors and other serious health problems [11,18,19].

There have been done several investigations about fungi inhibition by lactic acid bacteria [20–22] but this study was done to assess the potentiality of LAB isolated from breast milk to inhibition of *A. flavus* growth.

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB)

In this study, bacteria were isolated from various sources of milk. Some samples from human breast milk and others from milk samples of horse, camel and cow were isolated in Yazd, Iran. Serial dilutions of samples were prepared with PBS buffer at $1/10^2$ up to $1/10^7$. To isolate lactic acid bacteria, the diluted samples were grown on Man-Rogasa-Sharpe (MRS) broth (QUELAB, Canada) (pH 6.5) overnight under anaerobic condition at 35°C. Then the bacteria from each of the dilutions were spread on MRS agar medium and the plates were incubated at 37°C for 48 h under anaerobic conditions [23].

Then the plates were overlaid with semisolid malt extract agar (0.7%) consisting of 5×10^5 spores per ml of *A. flavus* and the plates were incubated at 30°C for 48 h in aerobic condition. Colonies of bacteria that show inhibition zone were selected for further investigation. The isolated lactic acid bacteria were stored at −20°C in MRS broth medium containing 50% glycerol. All dilutions and medium were do up freshly with double distilled water. IP, AC, FA [11,24]. LAB isolates were identified by cell and colony morphology, gram and spore staining and biochemical tests: catalase and motility test and growth at 15°C and 45°C [11].

Preparation of spore suspension of A. flavus

The mold strain used in this study was *A. flavus* PTCC 5004. This strain was obtained from Iranian Research Organization for Science and Technology (IROST, Iran).*A. flavus* was incubated on potato dextrose agar (PDA) (SIGMA-ALDRICH) slant tube at 30°C. Spores of *A. flavus* were collected from 7-days-old PDA tube cultures with sterile water containing 0.1% (v/v) tween 80 (Merck), and spores was separated by 15min shaking. The spore suspensions was adjusted to 5×10^5 spores/ml by using of Neubauer – Improved slide (HBG, Germany) [21,24].

Evaluation of LAB isolates influence on biomass production of A. flavus in MRS medium

Fresh cultures of isolated LAB were prepared and then 1000 µl of any culture was adjusted with PBS buffer to get OD 0.8 at 600nm. Then this bacterial suspension was mixed with 200 µl spore suspension of *A. flavus* in 15 ml of MRS broth medium. These cultures were incubated for 10 days at 30°C. Thereafter, filtration was performed on filter paper (Whatmann. No1) in order to separate mycelium from the medium and then it was completely washed with ethyl acetate, placed in an oven at 60° C until fixed weight was achieved and dry mass was calculated. Culture of the *A. flavus* without LAB bacteria was used as the control[24]. This study was performed in triplicate.

Determination of inhibitory activity of LAB bacteria against A. flavus on PDA medium

With 200 µl of each isolated LAB bacterial culture was made pour plate on PDA medium and was incubated at 30°C. After 3 h incubation, PDA disk of one week old mycelium of *A. flavus* with 0.5 cm diameter was placed in the middle of the PDA medium plate and was incubated at 30°C in the dark. The diameter of the fungi colony was measured for 2 weeks in order to estimate linear growth of *A. flavus*[24]. This study was performed in triplicate.

Dual culture overlay assay to determine inhibition of A. flavus by LAB isolates

The dual agar overlay assay with some modification was done to determine inhibitory activities of seven isolated LAB against *A. flavus*. Bacteria were spread on MRS agar plates and then incubated over night at 30°C in anaerobic condition. Then the plates were overlaid with semisolid malt extract agar (0.7%) containing 5×10^5 spores/ml of *A. flavus*. After aerobic incubation at 30°C for 24 and 48 h, the inhibition values were estimated. The inhibition value was specified as −, no inhibitory effect; +, poor inhibitory effect; ++, average inhibitory effect; +++, high inhibitory effect. Culture of the *A. flavus* without LAB bacteria was used as the control [11,24,10]. This study was performed in triplicate.

Inhibitory effect of LAB cell-free supernatants (CFS) against mycelia growth

This method was done to determine inhibitory activity of LAB isolates supernatants against *A. flavus* mycelia. At first, culture supernatants of isolated LAB were prepared by growing each isolates in MRS broth medium

anaerobically at 30°C for 24 h. For bacterial cell removing, the cultured medium was centrifuged at 6,000 rpm at room temperature for 15 minutes. Then the culture supernatants were filtrated by a 0.45 µm pore size filter. The supernatants were stored at 8°C until it was analyzed for antifungal activity and their compounds. 10 mL of supernatants were transferred in 50 mL flasks and inoculated with 200 µl spore suspension of *A. flavus* consisting 5 × 10^5 spores/ml of *A. flavus*. After culture incubation at 30°C for 24, 48 and 72 h, the mycelia growth was determined by measuring optical density (OD) at 560 nm. Culture of the *A. flavus* without LAB bacteria was used as the control [25,26]. This study was performed in triplicate.

Analyzing of CFS of LAB isolates

CFS of LAB isolates were prepared according to the method described before. The supernatants were analyzed for antifungal compounds, including organic acids and hydrogen peroxide and the pH was measured by a Metrohm 691 pH/ion Meter.

Determination of organic acids

High performance liquid chromatography (HPLC) was used to determine organic acids contents of LAB isolates CFS [27]. The HPLC system (Varian, USA) consisted of a pump (pump model: 210), an injection system (Auto sampler, model 410) with a 50µL injection loop, a Diode Array Detector (DAD, Model 3262) set at 210 nm and a column (Aminex® HPX-87H Ion Exclusion Column). The experimental conditions were controlled with Varian Prostar Station software. The mobile phase consisted of H_2SO_4 0.001 N (pH 2.27). The flow rate was 0.5 mL/min and the injection volume was 10 µL. The analysis was carried out at room temperature.

Determination of hydrogen peroxide

25 mL of diluted sulfuric acid was added to 25 mL of the CFS. Then titration was performed with 0.1 N potassium permanganate. Each milliliter of consumed potassium permanganate was equivalent to 1.07 mg of hydrogen peroxide. In this reaction, H_2O_2 reduced the permanganate to a colorless form. The bleached sample was considered as the end point [28].

Identification of lactic acid bacteria

16s rDNA sequence analysis was done to identify seven isolates of LAB from breast milk. Bacterial DNA was extracted from each of isolated LAB using DNA extraction kit (Sinagen, Iran). PCR reaction was done to amplify 16s rDNA using two primers. Forward primer (F 27): 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer (R 1492): 5'-GGTTACCTTGTTACGACTT-3' (Bioneer, Korea).

PCR amplification was carried out by a DNA thermal cycler, Flex Cycler. PCR conditions was 95 ◦C for 5 min, followed by 30 cycles of 94 ˚C for 90 s, 55 ˚C for 60 s, and 72˚C for 90s, and then 72˚C for 10 min for final extension. The sequence of each 16s rDNA was determined (1st base,Malaysia). The approximately 1450 bp 16s rDNA sequences of the seven isolates of LAB were edited using chromas software (edit sequence, DNA man). After that, the public databases were searched for finding the closest matching sequence for identification of the LAB isolates. The phylogenetic tree of RS1 and RS2 strains was constructed using the MEGA 6.0 program [13,24].

RESULTS

LAB isolation

23 LAB isolates were gained from the indigenously milk but seven of them showed good antifungal ability against *A. flavus* and were selected for this study. They were named as RS1, RS2, RS3, RS4, RS5, RS6 and RS7. The results of biochemical test for each LAB isolates are shown in Table 1.

Table 1. Characteristics of lactic acid bacteria having high antifungal activity

Strains	Source	Species	Accession Number	Shape	Gram reaction	Catalase test	Spore	Motility	Temp (°C)	
									15	45
RS1	Human breast milk (1)	*P. acidilactici*	KX611572	Cocci	+	-	-	-	+	+
RS2	Human breast milk (2)	*L.plantarum*	KX611571	Bacilli	+	-	-	-	+	+
RS3	Human breast milk (3)	*P. acidilactici*	KX611570	Cocci	+	-	-	-	+	+
RS4	Human breast milk (4)	*P. acidilactici*	KX611573	Cocci	+	-	-	-	+	+
RS5	Cow's milk	*L.plantarum*	KX611568	Cocco bacilli	+	-	-	-	+	+
RS6	Camel's milk	*P. acidilactici*	KX611574	Cocci	+	-	-	-	+	+
RS7	Hourse's milk	*L.plantarum*	KX61175	Bacilli	+	-	-	-	+	+

The effect of LAB isolates on biomass production of A. flavus in MRS medium

The dry mass of *A. flavus* in liquid MRS medium in paired cultures with LAB isolates in compare with the control (culture of the *A. flavus* without LAB isolates) is shown in Fig. 1. The biomass increase of paired cultures after 72 h was lower than the control in all of the samples. The decrease percentages of the biomass in paired cultures were 90% in RS1 isolate, 80% in RS6 isolate, 60% in RS2, RS4, RS5 and RS7 isolates, and 40% in RS3 isolate.

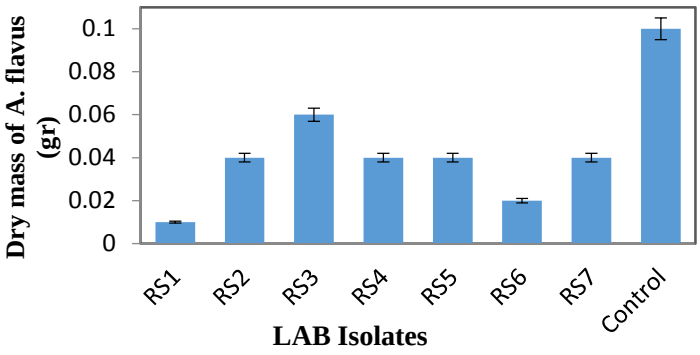

Figure 1. The effect of LAB isolates on Biomass of *A. flavus*. (The dry mass decrease percentages were 90% in RS1, 80% in RS6, 60% in RS2, RS4, RS5 and RS7, and 40% in RS3)

Determination of inhibitory activity of LAB isolates against A. flavus on PDA medium

The selected LAB isolates in paired cultures were able to inhibit the growth of *A. flavus* mycelia in solid PDA medium. However among them inhibitory ability of RS3 isolate was better than the rest. The findings of *A. flavus* mycelium growth in the paired cultures of LAB isolates on PDA medium are shown in Fig 2. RS3 isolate decreased the percentages of the mycelium growth 77.77% after 24 hour, 83.33% after 48 hour and 83.33% after 72 hour.

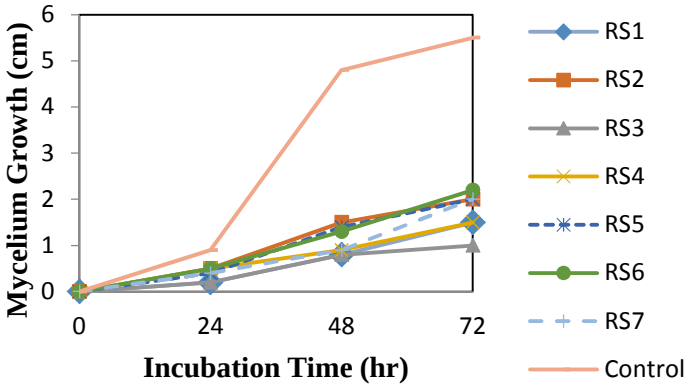

Figure 2. Mycelia growth of *A. flavus* on PDA in mixed culture with LAB isolates. (RS3 isolate decreased percentages of mycelium growth 77.77% after 24 hour, 83.33% after 48 hour and 83.33% after 72 hour)

Dual culture overlay assay to determine inhibition of A. flavus by LAB isolates

Growth of the *A. flavus* was inhibited by all of the seven LAB isolates in dual culture overlay assay. The results are shown in table 2. LAB isolates RS1, RS3, RS4 and RS6 had high activity between (+++ & ++) against of *A. flavus* and the isolates (RS2, RS5 and RS7) had moderate inhibitory activity against *A. flavus*.

Table 2. Inhibitory effect of LAB isolates against *A. flavus*.

(−, no inhibitory effect; +, poor inhibitory effect; ++, moderate inhibitory effect; +++, high inhibitory effect)

Strains	24 h	48 h
RS1	+++	++
RS2	++	++
RS3	+++	++
RS4	+++	+
RS5	++	+
RS6	+++	++
RS7	++	++
Control	-	-

Effect of CFS of LAB against mycelia growth of A. flavus

This method was done to assess the antifungal ability of the CFS of the seven LAB isolates culture against *A. flavus*. As shown in Fig. 3 the supernatant of RS1, RS2, RS3, and RS4 showed high prevention against *A. flavus* growth and among them, RS2 showed the best activity. The supernatant of other LAB isolates had moderate inhibitory activity on growth of *A. flavus*.

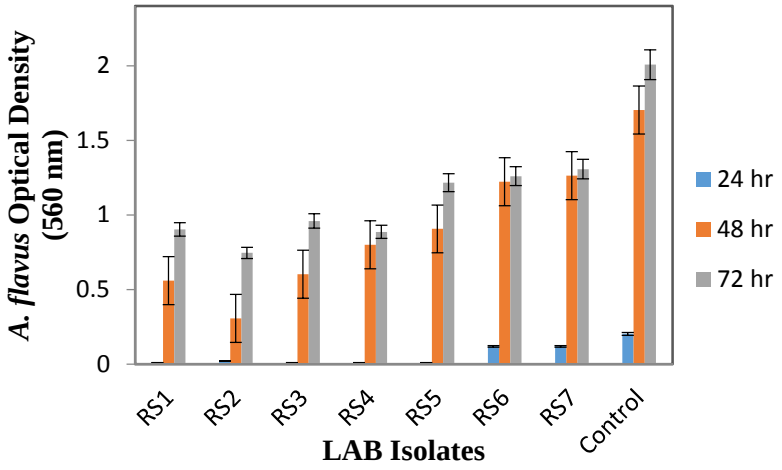

Figure 3. Antifungal activity of LABs supernatants against *A. flavus* (The CFS of RS2 showed the best activity against *A. flavus* growth at 24, 48 and 72 hours)

Table 3. The retention times of the test samples in comparison with standard solutions

Organic acids	Standard solution	RS1	RS2	RS3	RS5	RS56	RS7
Oxalic acid	8.87 ± 0.07	8.90	8.92	8.85	8.83	8.84	8.81
Lactic acid	15.46 ± 0.04	15.45	15.47	15.47	15.43	15.46	15.42
Formic acid	17.16± 0.06	-	-	17.14	17.15	-	17.11
Acetic acid	18.45± 0.1	18.55	18.55	18.55	18.53	18.55	18.52
Propionic acid	21.85± 0.03	-	21.85	-	-	-	-

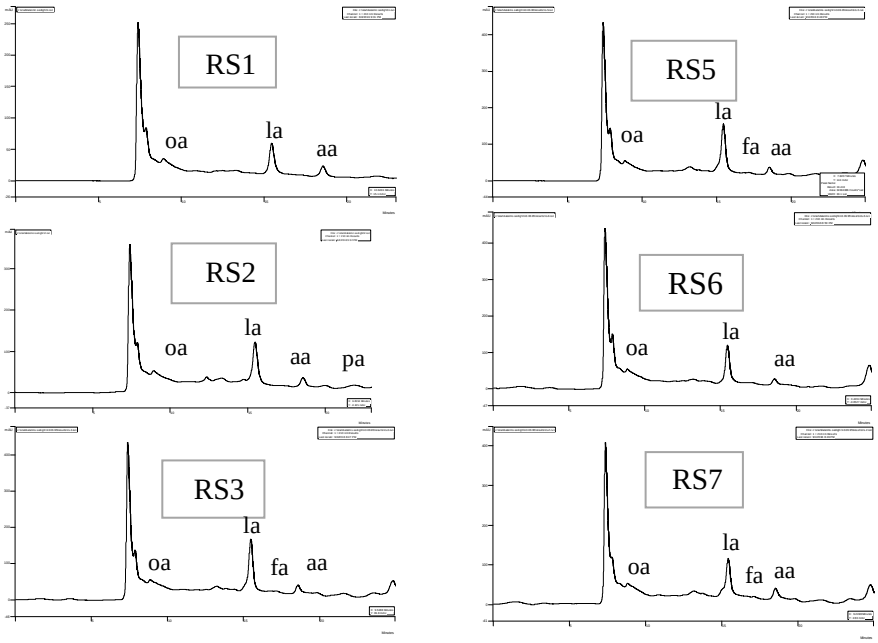

Figure 4. Chromatograms of CFS of LAB isolates performed with a columns containing H_2SO_4 0.001 N (pH 2.27) as mobile phase and at flow rate of 0.5 mL/min. (The x-axis shows the retention time of the organic acids compounds in minutes and the y- axis refers to the concentration (mAU). Peaks: (oa) oxalic acid; (la) lactic acid; (fa) formic acid; (aa) acetic acid; (pa) propionic acid)

Analyzing of CFS of LAB isolates

Contents of organic acids in the CFS of the isolated LAB were identified via comparison of their retention times with the standards in HPLC assay. The retention times for observed peaks of the standard solutions and the test samples are shown in Table 3 and the CFS chromatograms of LAB isolates are shown in Fig. 4.

Quantification was done based on the peak area on the linear calibration plots against concentration. The results are presented in Table 4. The amount of lactic acid in the CFS of RS2 strain was greater than others and there was a significant amount of propionic acid (17 mg/mL) in the CSF of RS2 strain. The concentration of hydrogen peroxide in the CFSs of the LAB isolates and the pH of the CFSs are presented in Table 4 too.

Table 4. Analyzing of CFS of LAB isolates

Organic acid (mg/mL)	RS1	RS2	RS3	RS5	RS6	RS7
Oxalic acid	0.05	0.21	0.45	0.23	0.08	0.10
Lactic acid	44.85	95.32	2.04	2.00	1.48	1.97
Formic acid	0	0	0.55	0.63	0	0.59
Acetic acid	0.28	0.34	0.54	0.30	0.25	0.65
Propionic acid	0	8.51	0	0	0	0
pH	4.5	5.5	4.5	5.6	4.7	5.5
Hydrogen peroxide (mg/mL)	13± 0.2	17± 0.2	12.5±0.1	15±0.1	12.8±0.2	14.8±0.1

Molecular identification of LAB isolates

The partial 16s rDNA sequence of each seven isolates was determined. Then public databases were searched to species identify of the seven isolates. The sequences of the LAB isolates, RS1, RS3, RS4 and RS6 displayed 99%, 98%, 97% and 97% similaritiesto the sequence of *Pediococcus acidilactici*. The sequences of the LAB isolates, RS2, RS5 and RS7 displayed 98% similaritiesto the sequence of *Lactobacillus plantarum*.

The partial 16s rDNA sequences of LAB isolates were submitted in GenBankwith a web-based sequence submission tool (GenBank: BankIt). The GenBank accession numbers for each nucleotide sequences are shown in Table 1. The phylogenetic tree of RS1 and RS2 strains is shown in Fig. 5.

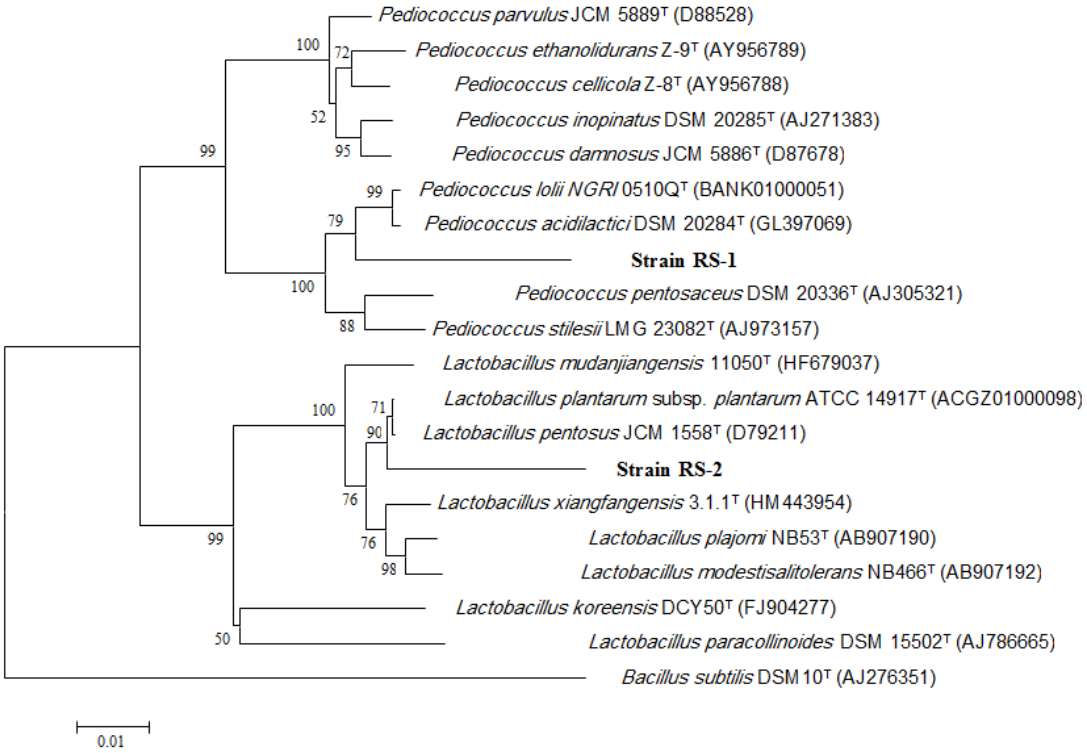

Figure 5. Phylogenetic tree of RS1 and RS2 strains. (Bold nodes indicate branches that were also obtained by minimum-evolution and maximum-likelihood methods. Numbers at nodes indicate levels of bootstrap support (%) based on analysis of 1000 resembled datasets; only values above 50 % are given. Bar, 0.01 substitutions per nucleotide position. The sequence of *Bacillus subtilis* DSM10^T was used as out group)

DISCUSSION AND CONCLUSION

Aspergillus flavus is one of the major causes of fungi food spoilage with important risks for human and animal health. Therefore, the prevention and control of its growth is important in the bio-preservation of food [12]. In this study the bio-preservation effect of isolated LAB from breast milk against mycotoxigenic *A. flavus* had been investigated. All the selected LAB strains in paired culture revealed the inhibitory ability against fungal mycelia growth in solid PDA and liquid MRS culture. LAB strains RS1, RS3, RS4 and RS6 showed high inhibitory activity against *A. flavus* in dual culture overlay assay. The CFS of RS1, RS2, RS3, and RS4 strains showed high prevention of *A. flavus* growth in broth medium.

In this research, the presence of several organic acids and hydrogen peroxide was confirmed in the CFS of the selected strains by HPLC and titration tests. Production of these organic acids led to acidification of the environment that commonly limits the growth of many pathogenic and spoilage microorganisms. A variety of organic acids produced by lactic acid bacteria have been used as fungal inhibitors, where it is believed that there are synergistic effects between them [9]. Organic acids also interfere in the electrochemical proton gradient and hydrogen peroxide oxidizes the membrane lipids that increase the permeability of the cell wall. These cases eventually leads to the destruction of the substrate transfer systems [29].

In this study, we found that there were significant amounts of propionic acid and H2O2 within the CFS of one of the strain. This can explain the good antifungal ability of CFS of RS2 strain. Hassan and colleagues (2015) reported that propionic acid had a fungicidal effect. However, calcium propionate exhibited no effect on growth of *A. flavus*[30]. León Peláez and colleagues (2012) reported that the different concentrations of lactic and acetic acid had antifungal activity against several strains of *A. flavus*. They showed that Lactic and acetic acid combinations had a synergistic effect and they increased the acid content of the medium and consequently decreased the growth rate of *A. flavus* and extended its lag phase [31]. Some study reported that, lactic and oxalic acids did not show any effect against *Fusariummoniliforme* growth, but propionic acid was the best organic acid that inhibited several species of fungi growth [32]. H2O2 also acts by membrane lipids peroxidation and cell membrane permeability that leads in the destruction of the substrate transport systems [29].

The identification results proposed that the seven isolates belonging to the Lactobacillaceae family as is shown in Fig. 5. *L. plantarum* strains are adaptable lactic acid bacteria that are detected in variety environmental niches andwerenoticed as super-star probiotic. *L. plantarum* has a historical background of natural incidence and safe use in many food products. Depending on their prevalent, easy growth and probiotic properties and human origin, various strains of *L. plantarum* have been examined for health efficacy [33]. Strains of *L. plantarum* are able to survive in gastrointestinal of mammalians [34]. Ingredients like hydroxyl fatty acids, cyclic dipeptides, phenolic compound and further metabolites obtained from *L. plantarum* have been examined for their antifungal potency. Utilize of *L. plantarum* strains to fungal growth reduction or inhibition has been very interesting applications due to the produced metabolites by LAB are remarked as natural preservatives [35]. Fernández and colleagues (2013) noted that *L. plantarum* was one of the main bacterial species isolated from human milk and its DNA sequences had been recovered from this biological fluid [4]. Gerez and colleagues (2009) reported that some of the *Lactobacillus spp.* including *L. plantarum* was able to inhibit the growth of spoilage molds that was found in small bakeries [36].

P. acidilactici is homo-fermentative bacterium that can grow in a various range of physiological conditions, so can colonize in the digestive tract [37]. *P. acidilactici* are considered by EFSA (European Food Safety Authority) to be proper for the Qualified Presumption of Safety (QPS) viewpoint to safety evaluation. So, *P. acidilactici* strains do not need any specific verification of safety [38]. This bacterium improves nutrition and growth of host. *P. acidilactici* secretes organic acids, decreases the pH level that prevents growth of some pathogens. It could be used in human beings food and animals feed against certain fungal pathogens [39].*P. acidilactici* has useful efficacy to its host via excitement the immune system. The probiotic *P. acidilactici* had been shown to be synergistic or antagonistic to rest of microorganisms with competition for adhesive places and generation a lot of bacteriocins [40].

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Conflict of interest
The authors declare that they have no conflict of interest.

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