Comp Clin Pathol (2017) 26:1083–1092
DOI 10.1007/s00580-017-2489-0

ORIGINAL ARTICLE

Inhibitory effects of lactic acid bacteria isolated from traditional fermented foods against aflatoxigenic *Aspergillus* spp.

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Received: 22 February 2017 / Accepted: 5 May 2017 / Published online: 27 May 2017

Abstract In the present study, after molecular identification of dominant lactic acid bacteria (LAB) isolated from traditional fermented foods, antifungal activity of the isolates was investigated against aflatoxigenic *Aspergillus* spp. Based on screening results, among the isolated LAB, *Pediococus lolii* had the highest inhibitory effect against *Aspergillus flavus* (70.32%) and *Aspergillus niger* (98.8%). Furthermore, antifungal activity of *P. lolii* stationary phase cell-free supernatant (CFS) was significantly ($P < 0.05$) higher than the effect of the logarithmic phase CFS. MIC values of *P. lolii* CFSs from logarithmic and stationary phases against *A. flavus* were 2 and 1%, and against *A. niger* were 4 and 1% (*v*/*v*), respectively. The media containing ≥4% of CFSs from logarithmic and stationary phases (*v*/*v*) had also totally inhibited from germination of *A. flavus* and *A. niger* spores. Safety assessment during 28-day oral administration of *P. lolii* revealed that there was no noticeable difference in specific growth rate, activity, behavior, hair luster, clinical chemistry, and hematological indices in treated rats in comparison to control group.

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Keywords *Pediococcus lolii* · Biopreservative activity · Aflatoxigenic *Aspergillus* · Cell-free supernatant

Introduction

Almost all animal feeds are potentially susceptible to toxigenic molds at any stage during storage, processing, packaging, and transportation. These types of molds produce mycotoxins that are highly toxic secondary metabolites (Rather et al. 2014; Quiles et al. 2016). Aflatoxins are one of the six major classes of mycotoxins produced mainly by *Aspergillus* species such as *Aspergillus flavus* and *Aspergillus niger* (Khanafari et al. 2007). Aflatoxin B_1 (AFB_1) is the most potent teratogenic, cancerogenic, and oestrogenic agent and a major risk factor for liver cancer (Kabak et al. 2009).

Consumption of contaminated feeds with aflatoxigenic *Aspergillus* spp. could have serious problem to public health. Therefore, effort to reduce of aflatoxigenic *Aspergillus* growth is serious concern around the world especially prevalent in developing countries. There are different physical, chemical, and biological methods for reduction of *Aspergillus* growth in contaminated animal feeds, but only a few of these methods have been accepted for practical use (Kohl et al. 2011). One of mentioned methods is inhibition of *Aspergillus* spp. growth by lactic acid bacteria (LAB) (Sangmanee and Hongpattarakere 2014). The use of LAB as a natural biopreservative is highly regarded because of their GRAS (generally recognized as safe) status (Belguesmia et al. 2013). In several studies, oral safety of LAB strains has been confirmed. Accordingly, these microorganisms can be used as safe biosupplements in food and pharmaceutical applications (Lara-Villoslada et al. 2007; Tsai et al. 2014; Damodharan et al. 2015).

LAB are a group of microaerophilic bacteria, Gram-positive, non-respiring, non-spore forming, catalase-negative,

cocci, or rod cells (Sangmanee and Hongpattarakere 2014). In recent years, various researchers have studied antifungal activity of LAB isolated from traditional fermented foods. In these studies, antifungal potential of *Lactobacillus* spp. (Sangmanee and Hongpattarakere 2014; Delavenne et al. 2015; Bian et al. 2016; Russo et al. 2016; Dong et al. 2017), *Enterococcus* spp. (Belguesmia et al. 2013), *Lactococcus* spp. (Roy et al. 1996; Varsha et al. 2016), and *Pediococcus* spp. (Dalie et al. 2010; Sadeghi et al. 2016; Sellamani et al. 2016) has also been described.

LAB isolates produce antifungal metabolites including 4-hydroxy-phenyllactic acid and phenyllactic acid (Lavermicocca et al. 2000), 3,6-bis(2-methylpropyl)-2,5-piperazinedion (Yang and Chang 2010), certain organic acids such as acetic acid, propionic acid, and lactic acid (Özcelik et al. 2016), hydroxy fatty acids (Sjogren et al. 2003), and cyclic dipeptides (Strom et al. 2002). These metabolites usually inhibit fungal spoilage by controlling the growth of toxigenic molds (Özcelik et al. 2016).

Due to the presence of LAB, traditional fermented products are usually less exposed to fungal spoilage. Therefore, microbial ecology of these products, especially non-aseptic fermented product ecosystems may be lead to identification of LAB isolates with unique antifungal capabilities. The objective of present study was to molecular identification and investigating the antifungal properties of native LAB isolated from traditional fermented products.

Material and methods

Indicator fungi

Aflatoxin producing *A. flavus* (PTCC 5004) and *A. niger* (PTCC 5154) were purchased from Iranian Research Organization for Science and Technology (IROST). These fungi were grown on yeast extract glucose chloramphenicol agar (YGC) (Merck, Germany) at 26 °C for 7 days. Fungal spores were harvested by addition of sterile Tween 80 solution (Merck, Germany) at 0.05% (*v*/*v*). Spore concentrations were adjusted to 10^4 spores/ml by a hemocytometer chamber (Wang et al. 2012).

Isolation and molecular identification of LAB

Ten different samples of traditional fermented products including Chal: fermented camel milk (four samples), whole barley sourdough (three samples), and whole wheat sourdough (three samples) were collected from rural area in north of Iran (Golestan Province). The samples were placed in a cooling box and transported to the Department of Food Science, Gorgan University of Agricultural Sciences and Natural Resources. Ten grams of each sample was stomachered in 90 ml of sterile ringer solution (Merck, Germany) with a Stomacher 400 (Seward, England) at 260 rpm for 3 min. An aliquot (1 ml) of the homogenate sample was spread plated onto MRS (de Man, Rogosa, and Sharpe) agar (Merck, Germany) and plates were then incubated at 37 °C in 10% CO_2 for 48 h. After incubation, colonies were streaked on MRS agar plates for obtaining single colonies. Then colonies were tested for Gram-stain morphology, catalase activity, and microscopic cell morphology (Yang et al. 2012).

For molecular identification, isolated strains were cultured in MRS broth at 37 °C in 10% CO_2 for 24 h, and DNA was extracted using the purification kit based on the manufacturer's protocol (GeneAll, Korea). 16S rRNA gene of isolates were subjected to PCR amplification with primers F44 (5′-RGTTYGATYMTGGCTCAG-3′) and R1543 (5′-GNNTACCTTKTTACG ACTT-3′) by a thermo-cycler (Corbett N15128, Australia). Optimized PCR reaction was carried out according to Yang et al. (2012) in 20-μl final volume including 10-μl Taq DNA polymerase master mix red (Ampliqon, Denmark), 1.5 μl of each primer at concentration of 0.5 mM, 5 μl ddH_2O, and 2 μl DNA with concentration of 100 ng/μl. Subsequently, PCR products (1500 bp amplicon) were electrophoresed in 1.5% (*w*/*v*) agarose gel in TBE buffer for 40 min at a constant voltage of 90 V and were visualized by UV transilluminator (Kiagen, Iran). Finally, the PCR products were sequenced by MWG Co. (Germany) and the sequencing results were checked by BLASTn procedure with available data in gene bank.

Antifungal activity of LAB isolates

Overlay method was used to investigate the antifungal activity of LAB isolates with slight modifications (Magnusson and Schnurer 2001). This method was performed using MRS agar plates (6 cm) on which LAB were inoculated as two 3-cm-long lines and incubated at 37 °C for 72 h in 10% CO_2. The plates were then overlaid with 15 ml of YGC agar containing 10^4 fungal spores/ml and incubated at 26 °C until the fungi growth was almost complete in the control plates. Inhibition zones in the pictures taken by a digital camera were measured by the ImageJ software (version 1.41).

Determination of logarithmic and stationary phases of LAB isolate

Overnight culture of the most effective LAB isolate was diluted to 10^3 CFU/ml in MRS broth and then incubated at 37 °C in 10% CO_2. Quantities of optical density (OD) of the isolate culture were monitored by spectrophotometer (LTD-T80T, England) at 600 nm at 1-h intervals, until receiving the growth curve to stationary phase (Gulahmadov et al. 2009).

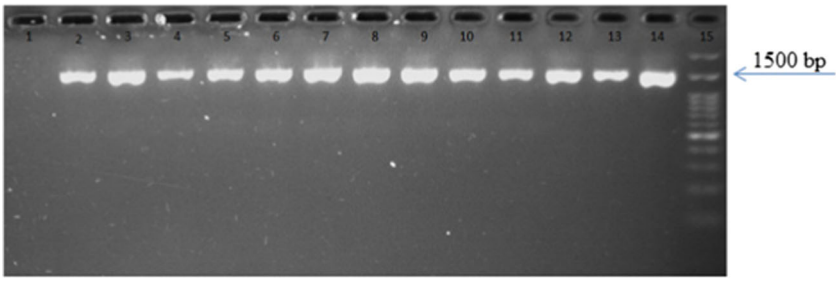

Fig. 1 Agarose gel electrophoresis of PCR products for the LAB isolates. *Lane 1* negative control, *lanes 2–14* extracted DNA from the LAB isolates (1500 bp), *lane 15* 100 bp DNA ladder

Preparation of cell-free supernatants (CFSs) from LAB isolate

After twice activation of each LAB isolate in MRS broth, CFSs from logarithmic and stationary phases were respectively prepared from culture collected 2 h before and after the end of the logarithmic phase. The supernatant was then centrifuged (Sigma 2-16 KL, United States) at 4 °C, 14,000 *g* for 5 min and subsequently filtered through sterile 0.22-μm syringe microfilters (Biofil, China) to obtain the untreated CFS (Wang et al. 2012).

Antifungal activity of CFSs

Antifungal activity of CFSs was tested as reported by Wang et al. (2012) with some modifications. Briefly, CFSs were mixed with YGC (45 °C) to achieve a final concentration of 1–10%. Fungal spores (3 μl of *Aspergillus* spore suspensions containing 10^4 spore/ml) were then spotted at the center of plates and incubated at 26 °C. The control YGC plate was CFS-free and containing 10% (*v*/*v*) of sterile distilled water that was inoculated with 3 μl of *Aspergillus* spores at the center. Antifungal activity of CFS was measured by the ImageJ software, until the fungi growth in the control plates was almost complete. The lowest CFS concentration that inhibited visible growth of *Aspergillus* was also defined as minimum inhibitory concentration (MIC).

Table 1 Molecular identification of the LAB isolates from "traditional fermented products" by 16S rRNA sequence analysis

Code	Isolate	Isolation source
KEC1	*E. hirae*	Chal
KEC2 and KEC3	*E. faecium*	Chal
KEC4	*L. frementum*	Chal
KEC5 to KEC7	*L. brevis*	Chal
KEC8 to EC12	*L. delbruki*	Chal
KEW13	*L. brevis*	Whole wheat sourdough
KEW14 and KEW15	*L. rhamnosus*	Whole wheat sourdough
KEW16	*L. aciduphilus*	Whole wheat sourdough
KEW17	*L. plantarum*	Whole wheat sourdough
KEW18 and KEW19	*E. faecium*	Whole wheat sourdough
KEW20	*E. hirae*	Whole wheat sourdough
KEW21	*L. coria*	Whole wheat sourdough
KEB22	*L. sakei*	Whole barley sourdough
KEB23	*L. reuteri*	Whole barley sourdough
KEB24 to KEB26	*L. rhamnosus*	Whole barley sourdough
KEB27	*P. lolii*	Whole barley sourdough
KEB28	*P. pentosaceus*	Whole barley sourdough

Preparation of activated *Pediococus lolii* for oral gavage in rats

For this purpose, the method of Tsai et al. (2014) was used. Briefly, bacteria grown in MRS broth were harvested by centrifugation (10,000 rpm for 5 min at 4 °C), then washed twice with phosphate-buffered saline (PBS), and resuspended in PBS to final OD of 0.8 (10^8 CFU cells/ml) at 600 nm, as measured by a spectrophotometer (LTD, England).

Safety assessment

Eighteen 4-week female Wistar rats were purchased from Pasteur Institute of Iran. Eighteen rats were then divided into two different groups for feeding and each rat was placed in a separate cage. For 28 days, the rats received orally/daily 100 μl of PBS containing 10^8 CFU of *P. lolii*, while control group received 100 μl PBS only. The rats were also given free access to food and drinking water and the housing conditions were 22 ± 3 °C with relative humidity of 60 ± 5% and a 12-h dark and 12-h light cycle. These animals were observed daily, and weight, behavior, activity, and hair luster were recorded. Specific growth rate (SGR) or average weekly weight gain (g) was calculated based on the following formula: $(W - W_0 / W_0) \times 100$. W and W_0 indicate the rat weight on the defined feeding days and on day 0, respectively. On day 28, all rats were sacrificed and blood samples were collected for laboratory analysis. Platelet counts, red blood cell counts (RBC), white blood cells counts (WBC), mean

Table 2 The percentage of *A. flavus* growth in the presence of the LAB isolates during 4 days

Code	Day			
	1	2	3	4
Control	12.27 ± 0.62^{a}	62.72 ± 0.39^{a}	91.8 ± 2.2^{a}	100 ± 0^{a}
KEC1	0.56 ± 0.26^{bc}	18.72 ± 2.38^{b}	59.55 ± 5.87^{b}	100 ± 0^{a}
KEC2	0 ± 0^{c}	0 ± 0^{c}	25.67 ± 9.31^{ef}	64.47 ± 6.22^{e}
KEC3	0 ± 0^{c}	0 ± 0^{c}	1.28 ± 0.54^{i}	46.32 ± 7.17^{f}
KEC4	0 ± 0^{c}	62.33 ± 0.55^{a}	90.6 ± 0.68^{a}	100 ± 0^{a}
KEC5	1.11 ± 0.49^{b}	65.91 ± 5.17^{a}	90.56 ± 1.44^{a}	100 ± 0^{a}
KEC6	0 ± 0^{c}	0 ± 0^{c}	39.45 ± 6.84^{cde}	77.85 ± 2.36^{cde}
KEC7	0 ± 0^{c}	0 ± 0^{c}	27.33 ± 2.22^{def}	65.84 ± 5.41^{e}
KEC8	0 ± 0^{c}	9.50 ± 0.94^{d}	51.22 ± 5.96^{bc}	100 ± 0^{a}
KEC9	1.21 ± 0.52^{b}	14.22 ± 3.18^{bc}	40.88 ± 7.27^{cd}	98.12 ± 1.88^{ab}
KEC10	0 ± 0^{c}	10.70 ± 2.77^{dc}	64.07 ± 6.05^{b}	100 ± 0^{a}
KEC11	0 ± 0^{c}	9.04 ± 2.67^{d}	42.84 ± 3.04bc	98.56 ± 1.44^{ab}
KEC12	0 ± 0c	6.91 ± 1.62^{d}	52.79 ± 5.58^{bc}	97.56 ± 2.44^{ab}
KEW13	0 ± 0^{c}	0 ± 0^{c}	16.29 ± 3.84f^{gh}	90.5 ± 1.04^{abc}
KEW14	0 ± 0^{c}	2.06 ± 2.06^{c}	15.82 ± 5.36f^{gh}	67.85 ± 2.46^{e}
KEW15	0 ± 0^{c}	0 ± 0^{c}	26.76 ± 6.01^{ef}	53.22 ± 2.03^{f}
KEW16	0 ± 0^{c}	6.29 ± 2.84^{dc}	22.27 ± 3.17^{f}	95.74 ± 4.26^{ab}
KEW17	0 ± 0^{c}	0 ± 0^{c}	5.960 ± 4.01^{hi}	84.54 ± 15.46^{bcd}
KEW18	0 ± 0^{c}	0 ± 0^{c}	22.54 ± 12.63^{f}	89.22 ± 10.77^{abc}
KEW19	0 ± 0^{c}	0 ± 0c	19.45 ± 2.22f^{gh}	40.59 ± 1.53^{g}
KEW20	0 ± 0^{c}	0 ± 0c	8.10 ± 2.23ghi	34.10 ± 5.80^{g}
KEW21	0 ± 0^{c}	0 ± 0c	7.60 ± 1.31^{ghi}	37.94 ± 0.6^{g}
KEB22	0 ± 0^{c}	0 ± 0^{c}	6.22 ± 6.22^{hi}	38.65 ± 3.71^{g}
KEB23	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{i}	32.25 ± 6.65^{g}
KEB24	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{i}	52.09 ± 5.19^{f}
KEB25	0 ± 0^{c}	0 ± 0^{c}	1.39 ± 0.58^{i}	72.39 ± 6.10d^{e}
KEB26	0 ± 0^{c}	0 ± 0^{c}	20.20 ± 4.30f^{g}	64.01 ± 5.29^{e}
KEB27	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{i}	29.68 ± 4.21^{g}
KEB28	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{i}	38.71 ± 2.40^{g}

Means within a column with the *same lower case letters* are not significantly different at $P < 0.05$

corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) were determined by Kavosh Laboratory (Gorgan, Iran). Furthermore, plasma levels of liver enzymes including alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were also determined (Tsai et al. 2014).

Statistical analysis

All experiments were performed in triplicate, and data were reported as mean ± standard error. LSD multiple comparison was also applied to obtain significance level ($P < 0.05$). All tests were analyzed using one-way analysis of variance (ANOVA) design with SPSS software (version 20).

Results

Molecular identification of LAB isolates

A total of 40 potential LAB strains were randomly isolated from fermented products on MRS agar; 28 of these isolates (12 isolates from Chal, nine isolates from whole wheat sourdough, and seven isolates from whole barley sourdough) were Gram positive, catalase negative, cocci or rod, and non-spore forming bacteria. Molecular identification of the isolates was done by specific PCR as described above. Fig. 1 shows the agarose gel electrophoresis of PCR products with a target gene of about 1500 bp. These 28 isolates were identified by sequencing of the 16S rRNA gene that showed 99% similarity with the corresponding species in the NCBI database (Table 1).

Table 3 The percentage of *A. niger* growth in the presence of the LAB isolates

Code	Day				
	1	2	3	4	5
Control	6.91 ± 1.59^{a}	35.77 ± 1.32^{a}	60.2 ± 1.29^{a}	91.13 ± 5.17^{a}	100 ± 0^{a}
KEC1	0 ± 0^{e}	0 ± 0^{h}	17.83 ± 3.27^{ghij}	37.123 ± 3.21^{ghij}	59.99 ± 2.81^{g}
KEC2	0 ± 0^{e}	3.58 ± 2.02^{efgh}	21.35 ± 3.54^{gh}	32.25 ± 5.33^{ijkl}	46.04 ± 4.52^{h}
KEC3	0 ± 0^{e}	6.84 ± 2.49^{ef}	22.34 ± 3.43^{fg}	36.44 ± 5.33^{hijk}	47.51 ± 5.48^{h}
KEC4	0 ± 0^{e}	0.09 ± 0.04^{h}	0.14 ± 0.035^{m}	2.63 ± 0.37^{pq}	28.49 ± 4.17^{ij}
KEC5	1.27 ± 0.17^{de}	19.82 ± 4.15^{cd}	38.00 ± 6.35^{de}	73.59 ± 2.09^{bc}	83.33 ± 0.32^{cd}
KEC6	1.84 ± 0.72^{cd}	19.00 ± 2.17^{cd}	48.61 ± 1.50^{c}	72.89 ± 4.44^{bcd}	82.71 ± 2.31^{cde}
KEC7	0 ± 0^{e}	0 ± 0^{h}	10.78 ± 1.14^{jk}	27.58 ± 2.72^{klm}	60.39 ± 2.12^{g}
KEC8	0 ± 0^{e}	6.25 ± 1.65^{efg}	44.40 ± 1.51^{cd}	63.71 ± 3.53^{def}	82.790 ± 3.34^{cde}
KEC9	0 ± 0^{e}	15.64 ± 1.70^{cd}	29.73 ± 1.84^{f}	55.51 ± 2.61^{f}	80.52 ± 5.63^{de}
KEC10	3.29 ± 2.06^{bc}	29.45 ± 1.68^{b}	54.89 ± 1.22^{b}	80.25 ± 2.27^{b}	95.06 ± 4.93^{ab}
KEC11	0 ± 0^{e}	19.55 ± 4.77^{cd}	54.06 ± 0.16^{c}	65.55 ± 2.39^{cde}	86.13 ± 0.20^{cd}
KEC12	0 ± 0^{e}	20.54 ± 4.31^{c}	39.56 ± 1.11^{de}	75.77 ± 2.66^{b}	89.36 ± 2.87^{bc}
KEW13	3.83 ± 0.27^{b}	13.5 ± 1.84^{cd}	23.94 ± 4.51^{fg}	42.22 ± 2.83^{gh}	65.07 ± 4.04^{g}
KEW14	0 ± 0^{e}	0 ± 0^{h}	2.34 ± 1.22^{lm}	6.68 ± 0.76^{opq}	26.96 ± 0.48^{ij}
KEW15	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{m}	14.31 ± 4.16^{no}	32.59 ± 3.68^{i}
KEW16	1.56 ± 0.73^{cde}	15.56 ± 1.44^{cd}	40.7 ± 4.66^{de}	58.89 ± 3.28^{ef}	74.17 ± 3.90^{ef}
KEW17	0 ± 0^{e}	0 ± 0^{h}	23.85 ± 1.87^{fg}	45.95 ± 4.07^{g}	100 ± 0^{a}
KEW18	0 ± 0^{e}	7.99 ± 2.12^{e}	14.45 ± 2.88^{hijk}	26.20 ± 1.64^{lm}	44.58 ± 0.60^{h}
KEW19	0 ± 0^{e}	0 ± 0^{h}	7.88 ± 3.21^{kl}	20.62 ± 4.26^{mn}	30.94 ± 3.96^{i}
KEW20	0 ± 0^{e}	14.47 ± 1.35^{d}	21.11 ± 3.82^{gh}	27.06 ± 5.69^{klm}	31.62 ± 3.50^{i}
KEW21	0 ± 0^{e}	2.37 ± 2.37^{fgh}	34.20 ± 2.49^{e}	40.65 ± 2.19^{ghi}	66.67 ± 0.45^{fg}
KEB22	0 ± 0^{e}	0 ± 0^{h}	19.51 ± 3.6^{1ghi}	28.83 ± 6.16^{jkl}	48.19 ± 5.71^{h}
KEB23	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{m}	10.11 ± 3.91^{op}	33.15 ± 1.61^{i}
KEB24	0 ± 0^{e}	0 ± 0^{h}	10.66 ± 1.76^{jk}	21.38 ± 3.48^{mn}	30.45 ± 0.58^{i}
KEB25	0 ± 0^{e}	1.77 ± 0.65^{fgh}	12.27 ± 1.7^{1ijk}	21.95 ± 3.72^{mn}	27.21 ± 2.83^{ij}
KEB26	0 ± 0^{e}	1.00 ± 0.47^{gh}	2.69 ± 2.69^{lm}	5.82 ± 4.56^{opq}	29.34 ± 0.92^{ij}
KEB27	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{m}	0 ± 0^{q}	1.2 ± 0.56^{k}
KEB28	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{m}	0 ± 0^{q}	25.79 ± 6.76^{ij}

Means within a column with the *same lower case letters* are not significantly different at $P < 0.05$

Antifungal activity of LAB isolates

Antifungal activity of LAB isolates is shown in Table 2 (against *A. flavus*) and in Table 3 (against *A. niger*). As it is indicated in Table 2, *A. flavus* filled the entire surface of the control plate at the end of the fourth day, while LAB isolates showed different inhibitory effects ranging from 0–53.68% (Chal), 4.26–65.9% (whole wheat sourdough), and 27.61–70.32% (whole barley sourdough). After 4 days, among the 28 isolates of tested LAB, eight isolates from Chal and three isolates from whole wheat sourdough had no inhibitory effect against *A. flavus*, while all isolated LAB from whole barley sourdough had significant effect on *A. flavus* growth ($P < 0.05$) in comparison to control plate.

Among the entire LAB isolates, *P. lolii* isolated from whole barley sourdough had the highest inhibitory effect (70.32% inhibition) against *A. flavus* (Fig. 2). In this study, we also observed that LAB isolated from traditional fermented products had proper inhibitory effect against *A. niger* growth. *A. niger* filled the entire surface of the control plate at the end of the fifth day, but growth of the mold in the presence of LAB isolated from Chal, whole wheat, and whole barley sourdoughs ranged from 28.49–95.06, 26.96–100, and 1.2–33.15% after 5 days, respectively (Table 3). According to our finding, LAB isolated from whole barley sourdough showed a broad spectrum of antifungal activity against *A. niger* and among the isolates, *P. lolii* (Fig. 2) and *Pediococus pentaseous* had respectively the highest inhibitory effects against *A. niger* growth with 98.8 and 74.21% inhibition at the end of the fifth day.

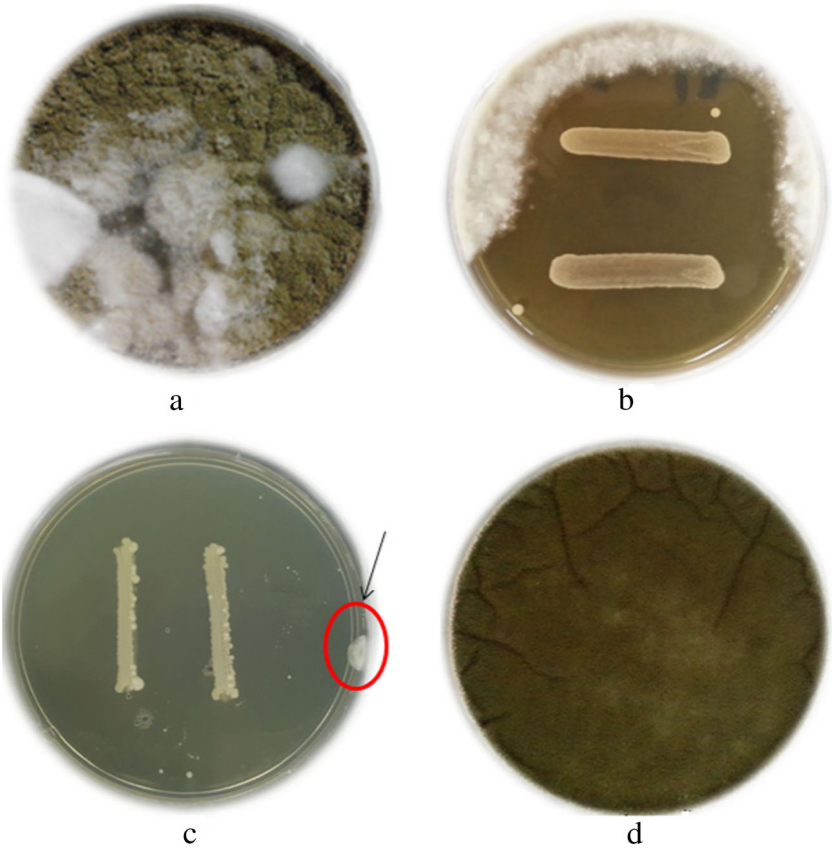

Fig. 2 Inhibitory effect of *P. lolii* isolated from whole barley sourdough after 4 days against *A. flavus* (**a**) and at the end of the fifth day (the *marked circle* show the fungus growth) against *A. niger* (**c**) compared with controls (**b** and **d**, respectively)

Antifungal activity of CFSs

Based on the results of overlay tests in the present study, *P. lolii* showed the highest antifungal activity against *A. flavus* and *A. niger* and so on. We focused on investigating the antifungal activity of *P. lolii* CFS. Stationary phase from *P. lolii* was started 10 h after incubation (data not shown). Therefore, CFSs from logarithmic and stationary phases were respectively prepared after 8 and 12 h after incubation. Table 4 shows

Table 4 The percentage of *A. flavus* growth in the presence of *P. lolii* CFSs

% CFS	*A. flavus* growth in the presence of logarithmic CFS	*A. flavus* growth in the presence of stationary CFS	*A. niger* growth in the presence of logarithmic CFS	*A. niger* growth in the presence of stationary CFS
0 (control)	$100 \pm 0A^{a}$	$100 \pm 0A^{a}$	$100 \pm 0A^{a}$	$100 \pm 0A^{a}$
1	99.13 ± 1.22^{Aa}	73.2 ± 7.59^{Bb}	100 ± 0^{Aa}	65.36 ± 4.12^{Bb}
2	67.4 ± 5.1^{Ab}	64.57 ± 10.38^{Abc}	99.7 ± 0.42^{Aa}	52.07 ± 5.13^{Bc}
3	60.53 ± 3.51^{Abc}	53.45 ± 8^{Ac}	99.4 ± 0.84^{Aa}	30.91 ± 9.12^{Bd}
4	48.55 ± 8.57^{Ad}	23.98 ± 7.62^{Bde}	44.81 ± 6.13^{Ab}	17.21 ± 5.58^{Be}
5	40.9 ± 5.05^{Ade}	14 ± 4.17^{Bef}	27.66 ± 3.02^{Ac}	12.26 ± 4.34^{Be}
6	31.44 ± 5.68^{Ae}	6.39 ± 3.68^{Bfg}	15.61 ± 4.17^{Ad}	0 ± 0^{Bg}
7	16.9 ± 5.72^{Af}	0 ± 0^{Bg}	11.4 ± 3.06^{Ad}	0 ± 0^{Bg}
8	2.06 ± 1.49^{Ag}	0 ± 0^{Ag}	0 ± 0^{Ae}	0 ± 0^{Ag}
9	0 ± 0^{Ag}	0 ± 0^{Ag}	0 ± 0^{Ae}	0 ± 0^{Ag}
10	0 ± 0^{Ag}	0 ± 0^{Ag}	0 ± 0^{Ae}	0 ± 0^{Ag}

Means within a column with the *same lower case letters* are not significantly different at $P < 0.05$

Means within a row with the *same upper case letters* are not significantly different at $P < 0.05$ (separately for each indicator fungus)

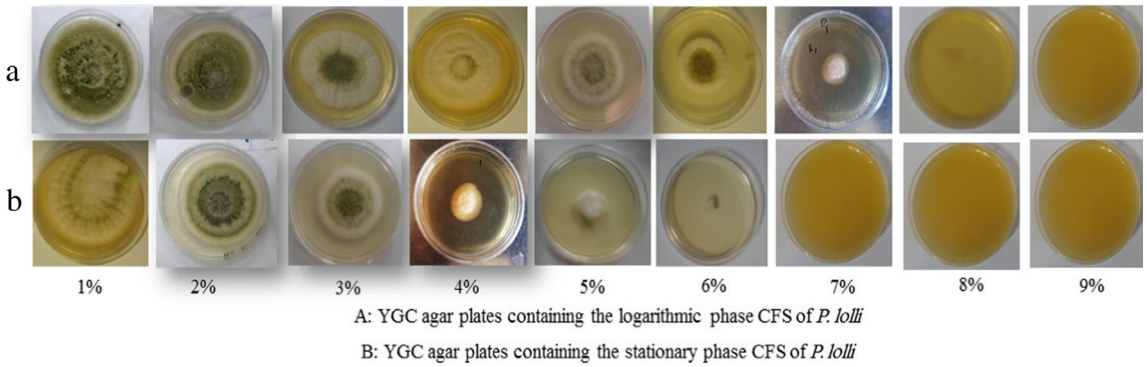

Fig. 3 Growth of *A. flavus* in the presence of *P. lolii* CFS

the antifungal activity of CFSs obtained from *P. lolii* against *A. flavus*. According to Table 4 data, the MIC values of *P. lolii* CFSs from logarithmic and stationary phases against *A. flavus* were 2 and 1% (*v*/*v*), respectively. The media containing 9% of logarithmic phase CFS and 7% of stationary phase CFS completely inhibited the growth of *A. flavus* mycelia.

The results indicated that antifungal activity of *P. lolii* stationary phase CFS was significantly ($P < 0.05$) higher than the effect of the logarithmic phase CFS. In addition, the media containing ≥4% of the stationary phase CFS (*v*/*v*) had totally inhibited from germination of *A. flavus* spores (Fig. 3).

MIC values of *P. lolii* CFSs from logarithmic and stationary phases against *A. niger* were shown in Table 4, too. MIC value of the stationary phase CFS was 1%, while for the logarithmic phase was 4%. Complete inhibition from *A. niger* growth was also occurred at 8% of logarithmic phase CFS and 6% of stationary phase CFS, respectively. Fig. 4 indicates that germination of *A. niger* spores was absolutely inhibited in the presence of 4% or more of the stationary phase CFS (*v*/*v*).

SGR, hematological, and clinical chemistry parameters

SGR of rats is shown in Table 5. Based on these results, during 28-day oral administration of *P. lolii*, the SGR increased in both control and treated groups but there was no significant difference ($P > 0.05$) between control and treated groups on the defined feeding days. Furthermore, any noticeable difference in the activity, behavior, and hair luster of the rats was observed in comparison to control group.

Clinical chemistry and hematological analysis data were also shown in Table 6. As it is indicated, in treated group, no significant change was observed in platelets, WBC, RBC, MCH, MCHC, MCV, and liver enzymes and all values were within normal physiological ranges.

Discussion

Modern agriculture and animal production systems need to reliable and safe methods to prevent and minimize *Aspergillus* growth rate. Inhibition of the mold growth by natural biological antagonists is a good example of these safe and efficient ways (Sangmanee and Hongpattarakere 2014). Furthermore, consumer demand for reduced use of antibiotics and chemical preservatives has led to more interest in biological preservation of foods and animal feeds in recent years. Among natural preservatives, LAB are special interest groups and their application has a long history in foods and animal feeds (Russo et al. 2016; Khanafari et al. 2007). Fermented

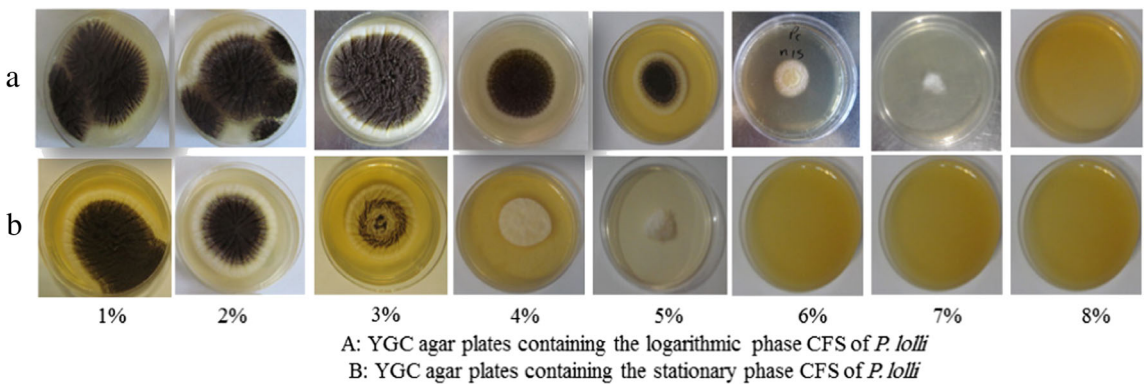

Fig. 4 Growth of *A. niger* in the presence of *P. lolii* CFS

Table 5 Specific growth rate of rats that were fed with *P. lolii* for 28 days

	Week 1	Week 2	Week 3	Week 4
Control group	20.98 ± 0.29^{Da}	53.78 ± 5.13^{Ca}	72.59 ± 4.37^{Ba}	99.96 ± 0.69^{Aa}
Treated group	20.54 ± 0.06^{Da}	57.34 ± 2.07^{Ca}	75.91 ± 0.23^{Ba}	102.1 ± 3.3^{Aa}

Means within a column with the *same lower case letters* are not significantly different at $P < 0.05$

Means within a row with the *same upper case letters* are not significantly different at $P < 0.05$

foods such as sourdough are the most common sources for LAB isolation. Based on competition ability and adaptability of predominant sourdough LAB to non-aseptic conditions of this fermented ecosystem, these LAB usually show strong antimicrobial activity (Sadeghi et al. 2016).

In the present study, we identified 28 LAB isolates by PCR method. Among those, 12 and nine LAB isolated from Chal and wheat sourdough belonged to the genera of *Lactobacillus* and *Enterococcus*, respectively. Moreover, seven LAB isolated from barely sourdough belonged to the genera of *Lactobacillus* and *Pediococcus*. Among mentioned isolates, *P. lolii* had the highest inhibitory effects against *A. flavus* and *A. niger*. According to these results, antifungal activity of *P. lolii* stationary phase CFS was higher than logarithmic phase CFS significantly ($P < 0.05$). Few studies have been conducted on *P. lolii* functional features. For example, Lee et al. (2012) analyzed the profile of organic acid in culture media of *P. lolii* by GC/MS and recently, Ju et al. (2016) confirmed the protective efficacy of *P. lolii* against influenza virus but there is no report about the antifungal activity of *P. lolii* until now. It should be noted that we identified *P. lolii* with strong antifungal activity from traditional whole barely sourdough for the first time.

Table 6 Clinical chemistry and hematological finding in rats that were fed with *P. lolii* for 28 days (mean ± SD)

Parameters			
Clinical chemistry	Control group	Treated group	Normal range*
Platelet (10^3/μl)	718.33 ± 64.59^{a}	763 ± 57.07^{a}	500–1300
WBC (10^3/μl)	7.43 ± 1.27^{a}	8.06 ± 0.89^{a}	6–18
RBC (10^6/μl)	6.34 ± 0.45^{a}	6.56 ± 0.32^{a}	6.76–9.75
MCH (pg)	21.35 ± 0.69^{a}	22.9 ± 1.14^{a}	Not determined
MCHC (%)	33.46 ± 0.44^{a}	34.07 ± 2.28^{a}	40
MCV (fl)	61.94 ± 1.59^{a}	64.19 ± 1.32^{a}	48–70
Hematological parameters			
ALT (IU/L)	51.48 ± 2.66^{a}	52.47 ± 0.93^{a}	17–77
AST (IU/L)	100.99 ± 2.43^{a}	95.31 ± 11.87^{a}	54–298
ALP (IU/L)	63.57 ± 4.56^{a}	62.81 ± 0.59^{a}	35–96

Means within a row with the *same lower case letters* are not significantly different at $P < 0.05$

*Research Animal Resources, University of Minnesota (http://www.ahc.umn.edu/rar/refvalues.html)

Digaitiene et al. (2012) identified five strains of antifungal LAB (*Lactobacillus sakei* KTU05-06, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9, and KTU05-10) with high activity against *Aspergillus*, *Fusarium*, *Mucor*, and *Penicillium*. Accordingly, antifungal LAB may be used as a suitable biopreservative to control the fungal spoilage in food and feed products. Antifungal activity of *L. rhamnosus* L60 and *L. fermentum* L23 was approved against aflatoxigenic *Aspergillus* spp. by Gerbaldo et al. (2012). *Lactobacilli* strains fully inhibited the *Aspergillus* growth of all strains assayed. Mentioned researchers have also concluded that mold contaminants of animal feeds could be significantly controlled by L60 and L23 strains. Rather et al. (2014) isolated *Lactobacillus plantarum* YML007 from Korean kimchi. *L. plantarum* YML007 and its supernatant delayed the growth of *A. niger*, *A. flavus*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Pichia membranifaciens* in maize. Furthermore, Wistar rats fed with supernatant treated maize showed more weight gain, compared with the control group. Based on these studies, antifungal activity of LAB is due to synergistic interactions between their metabolites such as weak organic acids, free fatty acids, cyclic dipeptides, piperidine derivatives, phenyl lactic acid, and phenolic compounds. Furthermore, recently, antifungal activity of cyclic dipeptides (Muhialdin et al. 2016), organic acids (Özcelik et al. 2016; Russo et al. 2016), and phenyl lactic acid (Russo et al. 2016) produced by LAB was approved. According to Strom et al. (2002), cyclic dipeptides are effective on quorum-sensing mechanism. Production of fungistatic bacteriocin-like substance by regulating signal peptides in LAB is a type of this quorum sensing. Free fatty acids, organic acids, and phenyl lactic acid also directly interact with the lipid bi-layer of fungi cell membrane and increase its fluidity. This interaction leads to change in membrane proteins, release of intracellular materials, cytoplasmic disorder, and finally cell destruction (Avis and Belanger 2001).

Some LAB strains produce piperidine derivatives (Li et al. 2012) and phenolic compounds (Annan et al. 2003) by several antifungal activities. Piperidine derivatives inhibit α-glycosidase activity, which plays a crucial role in gene regulation corresponding to carbohydrate metabolism in *Aspergillus* (Kato et al. 2002). Phenolic compounds can also interact with free radicals and prevent from formation of

hydroperoxy molecules (Annan et al. 2003), which are essential for both sexual and asexual body development of filamentous *Aspergillus* spp. (Calvo et al. 2001).

In the present study, we also observed that oral administration of *P. lolii* led to significant increase in the SGR of rats. Moreover, hematological and clinical chemistry parameters of the rats did not differ significantly from those of the control group. Over the past years, various researchers have reported safety of LAB isolated from traditional fermented foods. In these studies, safety of *Pediococcus* spp. (Tsai et al. 2014), *Lactobacillus* spp. (Sulemankhil et al. 2012; Jones et al. 2012; Szabo et al. 2011), and *Enterococcus* spp. (Tsai et al. 2004) has also been described. They reported that LAB isolates had no adverse effects regarding the behavior, activity, hematology, clinical chemistry indices, and growth rate of rats in comparison to control group.

Conclusion

In conclusion and based on our results, by considering the proper antifungal activity of *P. lolii* isolate and its CFS against aflatoxigenic *Aspergillus* spp. and approval of the isolate safety, it is possible to use from mentioned LAB and its CFS as biopreservative agents in processing of different foods and feeds.

Compliance with ethical standards

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

Funding This work is part of a PhD thesis supported by Gorgan University of Agricultural Sciences and Natural Resources and the authors thank the financial support.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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