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Original article

EVALUATION OF SAFETY AND ANTIFUNGAL ACTIVITY OF *LACTOBACILLUS REUTERI* AND *PEDIOCOCCUS DIACETILACTIS* ISOLATES AGAINST AFLATOXIGENIC *ASPERGILLUS FLAVUS*

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Summary

Sadeghi, A., M. Ebrahimi, B. Sadeghi & S. A. Mortazavi, 2019. Evaluation of safety and antifungal activity of *Lactobacillus reuteri* and *Pediococcus diacetilactis* isolates against aflatoxigenic *Aspergillus flavus. Bulg. J. Vet. Med.,* **22**, No 2, 190–199.

Biocontrol of moulds by lactic acid bacteria (LAB) instead of antibiotics and chemical preservatives is a new approach in veterinary medicine. The aims of present research were to perform molecular identification of dominant sourdough LAB isolates and to evaluate their *in vivo* safety and *in vitro* antifungal properties for using as biopreservative agents. Sequencing results of PCR products led to identification of *Lactobacillus reuteri* and *Pediococcus diacetilactis* as LAB isolates. Antifungal activity of the isolates and their cell-free culture filtrate (CCF) were also confirmed against aflatoxigenic *Aspergillus flavus*, respectively by overlay and spore spot methods. Accordingly, antagonistic effect of *P. diacetilactis* and its CCF were significantly ($P<0.05$) higher than *L. reuteri* and CCF of mentioned LAB isolate. Clinical chemistry and haematological findings in mice fed LAB demonstrated also insignificant difference vs control mice and were in the normal range, which confirmed the safety of LAB isolates. By considering the importance of safe, food grade biocontrol agents, *L. reuteri* and *P. diacetilactis* isolates and their CCF may be considered as an alternative for antibiotics and other chemical preservatives in food and feed processing chain.

Key words: antifungal effect, biocontrol, lactic acid bacteria isolates, safety

INTRODUCTION

Contamination of food and feed ingredients with aflatoxigenic *Aspergillus* is an important public health problem and also leads to huge economic losses. These moulds are notable as indicator fungi in cereal based products and therefore, their

control before entrance in the human food chain is necessary. Furthermore, most of alternative treatments instead of antibiotics for resolving these infection agents in veterinary medicine are not sufficient, yet (Tell, 2005; Dalié *et al*., 2010).

The usage of lactic acid bacteria (LAB) as one of the best solutions for biocontrol of moulds has been indicated instead of antibiotics and chemical preservatives (Schnürer & Magnusson, 2005; Ryan *et al*., 2008). LAB are the most abundant microorganisms isolated from a variety of fermented foods and their metabolic activity during fermentation, influence the quality and safety characteristics of the products. These bacteria may also produce several bioactive compounds as biopreservative agents with a proper activity, against different pathogens (Messens & De Vuyst, 2002; Rouse *et al*., 2008).

Sourdough in comparison to other fermented food ecosystems commonly is a better candidate for isolation of LAB with antimicrobial abilities. Sourdough is prepared from non-aseptic fermentation of cereal flour or other cereal ingredients with water and so on especially the dominant LAB in these conditions can overcome to present pathogenic microorganisms due to their proper antimicrobial activity (Vogelmann & Hertel, 2011; Sadeghi *et al*, 2016). There are several reports about fungistatic or fungicidal effects of sourdough LAB isolates (Fazeli *et al*., 2004; Hassan & Bullerman, 2008; Coda *et al*., 2011; Garofalo *et al*., 2012; Axel *et al*., 2016). Based on the results of mentioned studies, antifungal metabolites produced by sourdough LAB and their reported mode of action include: acetic acid (intracellular acidification and inhibition of glycolysis), lactic and propionic acids (intracellular acidification), caproic acid (alters fungal membrane permeability), formic acid (inhibition of electron transport chain and uncoupling of phosphorylation), butyric acid (reduces colonisation), phenyllactic acid and its corresponding hydroxy derivate (inhibitor of phenylalanine dehydrogenase and inhibitor of spore synthesis), monohydroxy octadecenoic acid (increase membrane permeability) and peptide mixtures and bacteriocin like products (unknown mode of action).

Use of antifungal LAB as a food grade biostrategy is also depending on safety of these microorganisms (Adams, 1999; Daniel *et al*., 2006). There are several reports about using sourdough LAB or their metabolites as biopreservatives in real situation in bread processing. For example, Gerez *et al*. (2009) used LAB with antifungal properties for prevention of bread mould spoilage and recently, Axel *et al*. (2016) used antifungal sourdough LAB as biopreservation tool in processing of quinoa and rice bread.

The aims of present research were to perform molecular identification of dominant sourdough LAB isolates and to evaluate their *in vivo* safety and *in vitro* antifungal properties with regard to their introduction as biopreservative agents in food and feed processing chain.

MATERIALS AND METHODS

Molecular identification of LAB isolates

For isolation of abundant LAB from sourdough (a mixture of whole wheat flour with sterile distilled water, dough yield 160, incubated at 37 ^{o}C for 24 h), the sourdough serial 10-fold dilutions were spread on MRS agar plate (Merck, Germany) for 48 h at 37 ^{o}C and subsequently obtained colonies were streaked to purity on MRS agar and incubated in mentioned conditions, again (Sadeghi *et al*., 2016).

Molecular identification of the LAB isolates was carried out after DNA extraction (Bioneer, AccuPrep K-3032 DNA extraction kit, South Korea) from their pure single colonies and amplification of target sequence (1500 bp) with species specific primers (F: RGTATYMTGGCTCAG and R: GGNTACCTTKTTACGACTT 5'-3') in optimised thermal cycling (Corbett N15128, Australia) stated by Abnous *et al*. (2009). After agarose gel electrophoresis, PCR products were sequenced (MWG, Germany) and results were compared with the available data in NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast).

Indicator mould and spore preparation

Aflatoxigenic *Aspergillus flavus* (ATCC 200026) was purchased from American type culture collection (USA) as a lyophilised vial and after activation on potato dextrose broth (Merck, Germany) at 26 ^{o}C for 48 h was cultured again on mentioned conditions and then stored at -80 ^{o}C in nutrient supplemented with 25% (v/v) glycerol (Magnusson *et al*., 2003). The number of fungal spores harvested from a one week incubated *A. flavus* pure agar culture by shaking with sterile distilled water was also adjusted using a cell-counting haematocytometer (Neubauer chamber, Merck).

Antifungal activity of the LAB isolates

Overlay method described by Magnusson & Schnurer (2001) with some modifications was used for investigation of antifungal activity of the LAB isolates. Briefly, each LAB isolate was inoculated as two 3 cm long lines in a petri dish of solidified MRS agar and then incubated at 37 ^{o}C for 72 h. The plates were overlaid with 1% potato dextrose agar (Merck, Germany) containing 10^4 fungal spores/ mL and incubated at 26 ^{o}C. Fungal growth was examined at an interval of 24 h. The control samples were prepared using the same procedure, but the LAB isolate was not inoculated in the plate.

Preparation of cell-free culture filtrate (CCF) from LAB isolates

After overnight incubation of each LAB in MRS broth at 37 ^{o}C, the fermentation culture was collected and subsequently centrifuged (Hermle Z-323K, Germany) at 4 ^{o}C, 14000 g for 5 min. Then for preparation of CCF, the supernatant was filtered using a 0.22 mm sterile micro filter (Biofil, China), (Yang *et al*., 2012).

Evaluation of antifungal activity of LAB CCF

The antifungal activity of LAB CCF was investigated using the Wang *et al*. (2012) method. Media containing 50% (v/v) CCF were inoculated in paper discs (6 mm), in which 3 µL of the test fungus spores were placed at the center. The control plates containing the media were mixed with MRS (50%, v/v) instead of CCF. Antifungal activity of CCF against indicator fungus was measured at 24 h intervals, until fungal growth in the control plate was almost complete. During this period, mycelial growths (mm) were measured and fungal growth area was determined by image J software in both control and treated plates.

Safety assessment of LAB isolates

Male mice (30±4 g) were prepared from animal center of Golestan University of Medical Sciences (Gorgan, Iran) and were quarantined for a week at controlled conditions before experiments. The mice were divided to three groups containing five mice and administered orally 100 µL ringer solution containing 10^8 CFU of LAB for four weeks. Group I was control

and administered only 100 µL Ringer solution. Mice of groups II and III were administered LAB isolates and specific body weight of mice was monitored every day. After finishing experiment, mice were killed and their blood was collected according to instructions of the care and use of laboratory animals (OECD, 1999). Haematological parameters including platelet, white blood cells counts (WBC), red blood cells counts (RBC), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and clinical chemistry parameters including activities of alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate aminotransferase (AST) as liver enzymes were evaluated by test kits prepared from Cavosh Ltd. (Iran) and finally, the results were reported as unit/liter (IU/L).

Statistical analysis

All the tests were performed in triplicate and results were presented as mean values ± standard deviation. Data were compared by the one way analysis of variance (ANOVA) and Tukey post hoc at $P<0.05$ statistical significance with the SAS-9.1 software.

RESULTS

Gel electrophoresis of PCR products (Fig. 1) verified the specific amplification of target sequence and comparison to Genebank database led to identification of *Lactobacillus reuteri* and *Pediococcus diacetilactis* as LAB isolates.

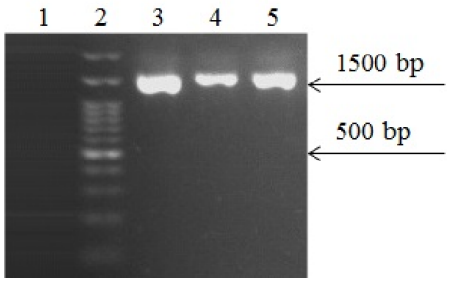

Fig. 1. Agarose gel electrophoresis of PCR products with 1500 bp target sequence. Lane 1: negative control (non DNA), lane 2: DNA ladder, lane 3: amplified DNA of *Lactobacillus* sp. as positive control and lanes 4, 5: amplified DNA of LAB isolates.

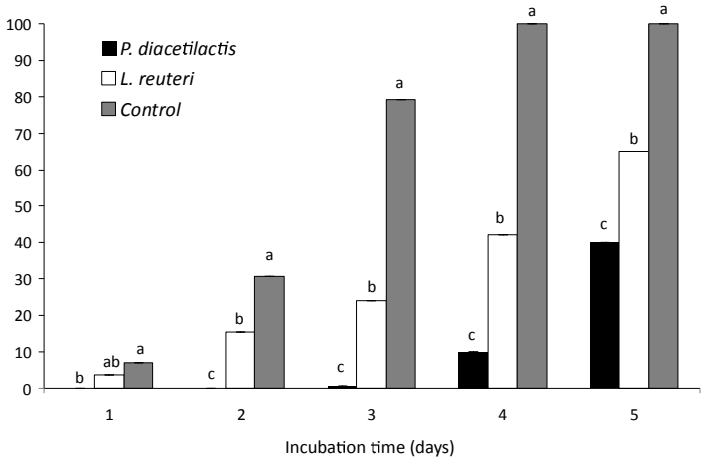

Fig. 2. Growth rate of *A. flavus* in presence of LAB isolates, during 5-day incubation in comparison to control sample.

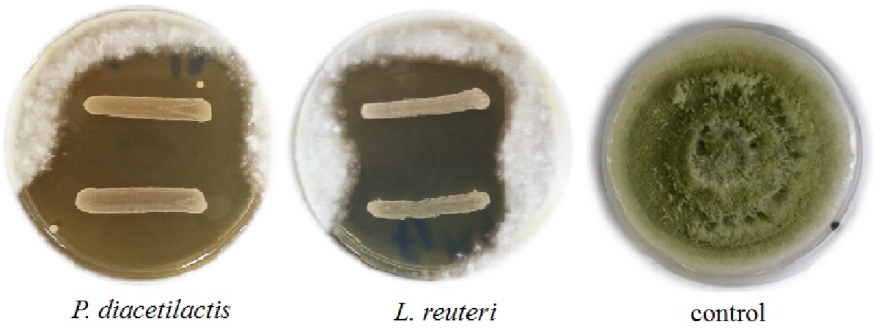

Fig. 3. Antagonistic activity of LAB isolates, against *A. flavus* after 5-day incubation in comparison to control sample in overlay assay.

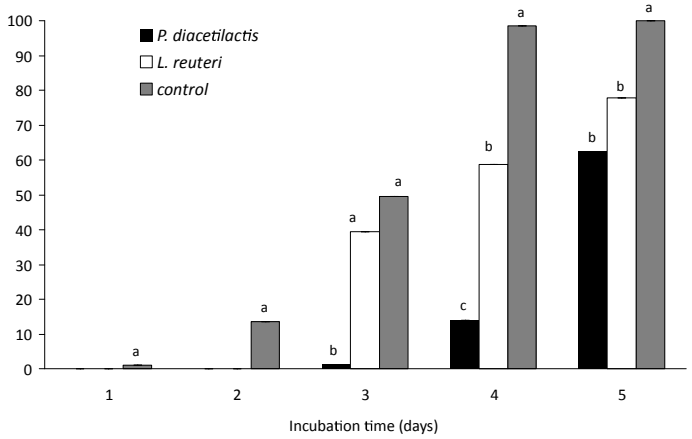

Fig. 4. Growth rate of *A. flavus* in presence of CCF obtained from LAB isolates during 5-day incubation compared to control sample.

According to the results of overlay assay (Fig. 2 and 3), antifungal activity of LAB isolates was confirmed in comparison to control sample. Furthermore, antagonistic activity of *P. diacetilactis* against *A. flavus* was significantly ($P<0.05$) higher than that of *L. reuteri*.

Results of antagonistic effect of LAB CCF against *A. flavus* showed that in almost all time intervals (except third day), diameter of *A. flavus* growth zone under treatment of LAB CCF, were significantly ($P<0.05$) lower than control sample. Based on this results, antifungal activity of P. diacetilactis CCF was significantly ($P<0.05$) higher than *L. reuteri* CCF (Fig. 4 and 5).

Body weight of mice was increased by administration of LAB with time. The highest and lowest body weights were measured for mice treated with *P. diacetilactis* and control, respectively (Table 1).

There was no significant difference ($P<0.05$) between haematological parameters including platelet, WBC, RBC, MCH, MCHC and MCV of mice treated with the

Fig. 5. Antifungal activities of CCF obtained from LAB isolates, against *A. flavus* after 5-day incubation in comparison to control sample.

Table 1. Specific growth rate of mice fed with LAB for 28 days

	Week 1	Week 2	Week 3	Week 4
Treated with *L. reuteri*	12.26±3.81[a]	18.37±7.13[b]	39.45±9.07[b]	63.15±9.63[ab]
Treated with *P. diacetilactis*	14.05±2.52[a]	33.66±6.55[a]	53.37±8.29[a]	77.24±8.6[a]
Control	8.32±4.58[a]	17.45±3.92[b]	25.31±5.64[c]	50.09±14.07[b]

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

Table 2. Clinical chemistry and haematological findings in mice fed LAB for 28 days

Parameters	Control group	Treated with *L. reuteri*	Treated with *P. diacetilactis*
Platelets (G/L)	801.0±14.0	794.5±44.5	795.0±26.0
WBC (G/L)	7.09±0.11	6.91±0.2	6.92±0.11
RBC (T/L)	6.37±0.17	6.24±0.12	6.33±0.17
MCH	22.45±2.05	22.50±1.50	23.22±0.23
MCHC (%)	31.45±0.55	32.99±0.12	32.08±0.92
MCV	62.85±1.15	61.65±0.65	63.35±0.54
ALP (IU/L)	65.03±3.14	68.07±1.06	67.19±3.79
ALT (IU/L)	49.23±1.99	51.58±1.66	49.76±1.70
AST (IU/L)	101.37±3.10	100.44±1.34	104.66±5.18

LAB isolates and control mice. Furthermore, ALP, ALT and AST activities of mice treated with the LAB isolates were insignificantly different vs control mice (Table 2). All ALP, ALT, and AST values measured for mice treated with the LABs were in the normal range, which is indicated that the hepatic cells were not damaged. The normal range for ALP, ALT and AST were 35–96, 17–77 and 54–298 IU/L, respectively (Giannini *et al*., 2005).

DISCUSSION

Our results confirmed the antifungal activity of *L. reuteri* and *P. diacetilactis* isolates and their CCF, against aflatoxigenic *A. flavus*. Antifungal activity of LAB is a complex hurdle and still partly unknown. Several studies have reported that the antifungal activity of LAB is not only relayed on the production of specific antifungal

compounds but also it is a combined effect of several other factors such as organic acids and low pH. The main antifungal compounds produced by LAB include fermentation products such as organic acids, proteinaceous substances such as antifungal peptides and low molecular mass inhibitory compounds such as cyclic dipeptides (Schnürer & Magnusson, 2005; Dalié *et al*., 2010). Due to the nature and mode of action, there are usually synergistically interactions between LAB antifungal metabolites such as pH-dependent compounds with types and amounts of produced organic acids. In addition to the pH effect, interaction between organic acids and other antifungal compounds such as proteinaceous metabolites with low pH optima of activity is important in complex nature of LAB antifungal hurdle (Magnusson *et al*., 2003; Hassan *et al*., 2015).

As mentioned, some of the antifungal inhibitory metabolites produced by LAB are proteinaceous in nature. Some investigators have reported the detection of antifungal heat stable small peptides with activity at low pH and totally inactivated by proteolytic enzymes. Furthermore, antifungal activity of bacteriocin like peptides such as pentocin and reuterin produced by LAB isolates has also been indicated (Magnusson & Schnurer, 2001; Rouse *et al*., 2008). The number of purified and characterised antifungal low molecular weight compounds is low but there are several reports about presence of these LAB metabolites. Cyclic dipeptides, phenyllactic acid and hydroxylated fatty acids are some of the most famous low molecular weight inhibitory compounds produced by sourdough LAB. A precise mode of action for antifungal metabolites can often not be defined due to a complex interaction between the different compounds and the synergistic effects among them (Lavermicocca *et al*., 2003; Black *et al*., 2013).

In the study of Fazeli *et al*. (2004), different concentrations of *L. casei*, *L. plantarum* and *L. fermentum* sourdough-isolated strains were used as sourdough starter cultures for Lavash bread processing. After a 20 h incubation at 30 ^{o}C, concentrations of 2% and higher of the three lactobacilli used in sourdough, delayed mould growth during Lavash storage. Accordingly, selected strains of lactobacilli have a crucial role in prevention of bread mould spoilage. Based on results of Hassan & Bullerman (2008), *L. paracasei* ssp. *tolerans* sourdough isolate was able to inhibit closely related mould species in the same genus to a similar degree and completely inhibited the growth of some *Fusarium* species compared to controls. Garofalo *et al*. (2012) confirmed *in vitro* antifungal properties of LAB sourdough isolates against fungal indicator cultures isolated from mouldy bread. Furthermore, the sourdough extracts prepared with the two most promising strains, identified as *L. rossiae* and *L. paralimentarius* revealed the occurrence of peptides as antifungal fraction in *L. rossiae* fermentation that also inhibited mold growth on panettone. Axel *et al*. (2016) investigated the production of carboxylic acids in quinoa and rice sourdoughs fermented with antifungal *L. reuteri* and *L. brevis* strains. In quinoa sourdough extract the greatest numbers of carboxylic acids at a much higher concentration were detected, among them, 3-phenyllactic acid and 2-hydroxyisocaproic acid were dominant. Furthermore, with the addition of *L. reuteri* and *L. brevis* (as bioprotective cultures) inoculated sourdoughs; respectively the shelf life was extended by 2 and 4 days for produced bread, that was similar to the chemically

acidified control. Accordingly, the preservation effect of the carboxylic acids had a minor effect on the antifungal activity in gluten-free breads.

The results of the present study showed that the use of *L. reuteri* and *P. diacetilactis* isolates offers a safe potential for their use as natural, food-grade biopreservative agents, too. Bernardeau *et al*. (2002) similarly reported that administration of *Lactobacillus rhamnosus* MA27/6B and *L. acidophilus* MA27/6 to mice significantly increased their body weight in comparison to control sample. Pavan *et al*. (2003) demonstrated that the administration of LAB not only did not cause damage to mice, but also acted as health-promoting factor.

CONCLUSIONS

Our results confirmed the safety of the LAB. Both isolates inhibited the growth of *A. flavus* mycelia. Because the conidia germination is most sensitive to inhibition, only some LAB can inhibit the growth of fungal mycelia while almost all of the strains are able to inhibit the conidial germination. By considering the importance of safe, food grade biocontrol agents, *L. reuteri* and *P. diacetilactis* isolates and their CCF may be considered as an alternative for antibiotics and other chemical preservatives. Meanwhile, use of results obtained from *in vitro* experiments with synthetic cultures in special conditions into the *in vivo* complex food systems is challenging, which needs further study. Furthermore, a re-investigation of these isolates is needed to better understand inhibitory mechanism against tested fungus.

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