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Screening of Lactic Acid Bacteria for Antifungal Activity against Fungi

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Abstract

Many chemical preservatives that target fungi growth in food have been approved and used for many years. Recently the consumers are looking and demanding for products without chemical preservatives and still maintain good shelf life and safe. The growth of spoilage fungi have been a global concern because of the economy loses and the health hazard of the mycotoxins produced by the spoilage fungi. A total of 22 lactic acid bacteria isolated from Tarhana and Lactic acid bacteria were screened for antifungal activity using dual agar overlay method and well method against *Alternaria alternata, Aspergillus parasiticus, Aspergillus oryzae Penicillium griseofulvum, Penicillium chrysogenum, Penicillium notatu, Penicillium citrinum, Penicillium roquefort, Aspergillus fumigatus*. Ten isolates showed inhibition activity after 72 h incubation at 30°C. Supernatant of 10 isolates with strong antifungal activity was evaluated by well method and they inhibited the growth of the fungi at 30°C for 72 h. F2,1 supernatant reduced the mass growth of *Penicillium griseofulvum, Penicillium chrysogenum Aspergillus fumigatus* and *Aspergillus parasiticus* when incubated for 6 days at 30°C. The isolates were identified using rapid ID 32 Strep as *Enterococcus durans* F2.1. F2.1 isolates studied inhibited the growth of the mycelia and conidia germination of the fungi which indicate the possibility of using LAB isolates as biopreservative.

Keywords: Antifungal; *Enterococcus durans*; Lactic acid bacteria

Introduction

Moulds are common spoilage organisms in different food and feed products. This spoiling moulds cause great economic losses worldwide. Food and feed contamination by fungi and by their mycotoxins constitute potential health hazard to consumers [1]. The inhibition of growth of fungi in foods remains a big challenge for food industries.

Many physical and chemical methods have been developed inhibiting of fungi for many years. Lactic acid bacteria contribute technological and nutritional properties and influence the food. They also extend the microbiological shelf-life of final products. Lactic Acid Bacteria (LAB) produce antimicrobial compounds which can be applied as food preservatives. Biopreservation refers to extended shelf-life and enhanced safety of foods obtained by the natural or added microflora or their antimicrobial products [1]. Lactic acid bacteria (LAB) have traditionally been used as natural biopreservatives in food and animal feed. Using microorganisms in order to control the fungi growth appears as a good alternative [2]. Lactic acid bacteria (LAB) have received much attention, especially because of their food grade and their GRAS status [3,4]. LABs preserving effect relates mainly to the formation of organic acids such as lactic and acetic acid and hydrogen peroxide, competition for nutrients and production of antimicrobial substances and bacteriocins, phenyllactic (PLA) and *p*-OH-phenyllactic acids (OH-PLA), caproic acid or reuterin [1,2,5].

The objective of this study is to evaluate the antifungal activity of LAB isolated from fermented food tarhana. Further, this study was to evaluate the behaviour of Lactic acid bacteria and fungi. The bacteria and their products can be applied as biopreservatives.

Material and Methods

Chemical materials

Malt extract agar (MEA), Lactobacillus Broth acc. to DE MAN, ROGOSA and SHARPE(MRS broth), Lactobacillus Agar acc. to DE MAN, ROGOSA and SHARPE (MRS agar) potato dextrose agar (PDA), Tween 80, NaCl, hydrogen peroxide, L-Arginine were purchased from Merck (Turkey). Czapek-Dox Agar and Yeast Extract purchased from Difco (Turkey). All other chemicals and reagents used were of analytical grade unless otherwise stated.

RiboPrinter® System dısposables (Sample carriers and buffer, Colony picks, Gel cassettes and membranes, Restriction enzymes–choose EcoRI, Lysing agents, Probes, including IS6110, Conjugate, base and substrate, DNA prep pack) were obtained from DuPont™ and ID 32 Strep and Blood agar plates were obtained from Biomeriux (Turkey).

Microorganisms

Twenty two LAB strains (F 5,3; F4,4; F5,4; F7,4; 2F3,2; F7,2; 2F5,7; F6,4,1; F6,2,3; F6,2,2,3; F6,4,2; F3,2; F7,8; F2,1; 2F2,2; 2F7,5; 2F4,1; F7,1,2; 2F4,2; 2F5,3) isolated from tarhana were obtained from the University of Anadolu, Faculty of Science (Eskişehir, Turkey). Frozen cultures of these lactobacilli were transferred into MRS broth (Merck, Darmstadt, Germany) using 1% inoculum and incubated at 30°C for 24 h. before use, they were subcultured at least twice.

Fungal cultures and preparation of the spore solution

The moulds *Penicillium roqueforti, Alternaria alternata, Penicillium notatum, Penicillium citrinum, Aspergillus parasiticus, Penicillium chrysogenum, Penicillium griseofulvum, Aspergillus fumigatus, Aspergillus oryzae (*University of Anadolu, Faculty of Science Eskişehir, Turkey) were used in this study for assay of antifungal activity *in vitro*.

Moulds were cultivated on Malt Extract Agar (MEA) (Merck,

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Darmstadt, Germany) at 25°C for 2 to 5 days and stored at 4°C. The fungi strains were grown on malt extract agar (MEA) at 25°C for 7 days. The conidia were collected in sterile Tween 80 (Merck, Darmstadt, Germany) at 0.05% (v/v) and counted at the microscope in a haemocytometer chamber. Collection fluid was used to adjust to 10^6 spores / mL.

Screening of bacteria for antifungal activity

Bacterial isolates, grown in 10 ml of MRS broth at 35°C overnight, were tested for their antifungal activity against fungi as an initial screening step. Two different assays, the overlay method and the dual-culture agar overlay method were employed to detect antifungal activity.

The antifungal activity of LAB was investigated with an overlay assay [6,7]. Bacteria were inoculated in 2-cm lines on MRS agar plates and allowed to grow at 30°C. Ten millilitre of soft (7%) malt extract agar containing 1 ml of inoculum of mould spore was then poured onto the agar plates and incubated at 30°C. After 48 h, the zone of inhibition was measured. The degree of inhibition was calculated as the area of inhibited growth.

Other method dual-culture agar overlay method, LAB strains were inoculated into MRS agar and poured plate and plates allowed growing at 30°C for up to 2-3 days in anaerobic jars. The plates then were overlaid with MEA (0.7%, 5 ml). Spotting 5 µl of containing about 10^5 spores of fungi per ml onto the surface of a MEA plate. After incubation for up to 9 days at 25°C, the plates were examined for the formation of inhibition zones around the bacterial colonies. The plates were examined for zones of inhibition in the test fungi.

Determination of inhibitory activity of cell-free supernatants

LAB was inoculated to an initial concentration of 10^6 cfu/mL in 50 mL of MRS broth (pH 6.5) and was incubated at 35°C for 48 h. Afterwards, broth culture was centrifuged at 5000×g for 15 min at 4°C and cell-free supernatants were filtrated (0.22 µm pore size filter; Millipore).

The Czapek-Dox Agar-Yeast Extract (Czapek Dox agar (Difco) w/ 1.0% yeast extract (Difco)) was rapidly dispensed in sterile petri dishes and, after solidification. Wells of 8 mm diameter were cork bored in the agar and a droplet of agar was added to each well. 80 µL of sterilized cell-free supernatants were dispensed in the wells and allowed to diffuse into the agar during a 2-h pre-incubation period at room temperature. Small portion of five day-old mycelia of fungi were placed in the middle of a Czapek-Dox Agar-Yeast Extract agar plate. Plates were incubated overnight at 30°C 9 d. Mycelia growth zone was measured by diameter.

Inhibition of tested moulds by *E. durans* F2.1 in MRS broth

The MRS broth was inoculated with 1% (v/v) fungi (10^5 spore/ml from with (treatment) and without (control) the addition of 1% v/v LAB (initial cell count in the inoculated MRS broth was approximately 10^6 cfu/mL). 20-mL inoculated MRS broth were incubated at 30°C for 5 days. Samples from each tube removed at different time and plated to count. Microbial counts, decimal dilutions were prepared in sterile 9 ml of 0.85% (w/v) NaCl (Merck, Darmstadt, Germany) and plated on media. The number of colony forming units (cfu/g) was determined by plate count procedure in MRS agar for LAB and potato dextrose agar (PDA) (Merck, Darmstadt, Germany) for enumeration of fungi. Plates were incubated at 30°C for 24 h, and then counted for viable organisms.

Identification of isolate F2.1

Isolate F2.1 were identified using the catalase, ammonia production from arginine, alpha hemolysis, beta hemolysis, growth at different temperatures (4, 10, 15 and 45°C), growth at different pH values, growth at different NaCl concentrations [8,9]. Carbohydrate fermentation tests were carried out using the rapid ID 32 Strep according to the manufacturer's instruction (BioMerieux, France). Automatic ribotyping was performed with a RiboPrinter® Microbial Characterization System (Qualicon Inc., Wilmington, DE)

Results and Discussion

Screening of the 22 LAB were performed against pathogenic fungi growth *in vitro*. Varying degrees of inhibition were detected against the moulds in the overlay method. *P. chrysogenum* was the most sensitive strain (Table 1). Ten of the 22 isolates showed good inhibitory activity against of the fungi in the overlay method. Of these 22 LAB strains, seven exhibited inhibitory effects tested fungi in dual-culture agar overlay methods (Table 2). *Alternaria alternata* was the most sensitive strain in dual-culture agar overlay methods.

Ten isolates (10/22) had inhibitory against spore germination and the mycelia growth of tested fungi (Table 3). LAB isolated from tarhana showed good inhibition activity against mycelia growth tested.

In this study we observed that the antifungal activity was strain-dependent as well as the evaluated fungal species and tested methods.

The growth of the mycelia and the conidia were inhibited by the supernatant of the LAB isolates by the well method. Growth of the mycelia was inhibited and there was no conidia forming observed from the survived mycelia.

L. rhamnosus was reported to inhibit the growth of *Aspergillus, Penicillium* and *Fusarium* [10]. Muhialdin and Hassan [11] reported that LAB isolated from fermented guava juice inhibited the spore germination and mycelia growth of *A.oryzae.*

From the total of LAB strains evaluated, F2,1 isolate was selected due to their inhibitory effect on all fungal strains tested. The F 2.1 isolate were identified using rapid ID 32 Strep as *Enterococcus durans* (Table 4) and it was confirmed by EcoRI ribotyping (Figure 1). *Enterococcus* spp. are rarely described to antifungal activities [12-14]. *E. durans* F 2.1 has antagonistic effects against tested fungal strains. *E. durans* A5-

Molds	Lactic acid bacteria	Antifungal activity
Alternaria alternata	F 7.4	+
Aspergillus fumigatus	F 7.4	+
Aspergillus oryzae	F 7.4 F 7.5 F 3.2	+ ++ +
Aspergillus parasiticus	F 2.1	+
Penicillium citrinum	F 7.4	+
Penicillium chrysogenum	F 7.2 F 5.4 F 6.4.2 2 F 7.5 F 2.1 F 3.2	+++ ++ +++ ++++ + ++ + ++
Penicillium griseofulvum	2 F 7.5 2 F 5.7	+++ +
Penicillium notatum	2 F 5.7	+
Penicillium roqueforti	F 7.2	++

(+)weak inhibition (İnhibition zone ≤ 0.5 mm), (++)modarate inhibition (İnhibition zone 0.6- 1.4mm)
(+++) İnhibition zone 1.5- 2.4mm, (++++) İnhibition zone .≥ 2.5mm

Table 1: Antifungal inhibition spectrum of LAB strains in a overlay methods.

Strains	*P. chrysogenum*	*A. parasiticus*	*P. griseofulvum*	*A. fumigatus*	*A. alternata*
F 5,3	-	+	++	-	-
F4,4	+	++	+++	++	-
F5,4	-	-	++	-	-
F7,4	+	++	++	++	-
2F3,2	+	-	++	++	-
F7,2	-	-	++	-	-
2F5,7	++	-	+	-	+
F6,4,1	+++	++	++	++	-
2F7,1	+++	-	++	-	-
F6,2,3	-	-	++	++	-
F6,2,2,3	-	-	++	++	-
F6,4,2	++	-	++	++	-
F3,2	-	+	++	++	-
F7,8	++	+	++	++	-
F2,1	-	+	++	++	-
2F2,2	++	-	++	-	-
2F7,5	-	+	+	++	-
2F4,1	-	+	++	++	-
F7,1,2	-	+	++	++	-
2F4,2	-	-	+	-	-
2F5,3	++	+	+++	-	-
2F1,2	+++	+	+++	+++	+

(-) No growth (+)a small growth(++)growth (+++) large growth

Table 2: Antifungal inhibition spectrum of LAB strains in a dual-culture agar overlay methods.

Tested Molds	Inhibition	Lactic Acid Bacteria									
		F5,4	F7,8	F7,2	2F5,7	F4,4	2F7,5	F3,2	F7,4	F2,1	2F3,2
A.parasiticus	Growth	++	++	++	+	++	++	++	++	++	++
	Spore	-	-	++	++	-	-	-	-	-	-
P.notatum	Growth	-	++	+	+	+++	+++	++	-	+	++
	Spore	++	-	++	++	++	++	++	++	++	++
P.roqueforti	Growth	+	++	+	+	+	+++	++	++	+	+
	Spore	++	-	-	++	++	++	++	++	++	++
P.citrinum	Growth	++	++	+	++	++	++	+	+	++	++
	Spore	-	++	++	-	-	-	-	-	++	++
P. griseofulvum	Growth	++	+	+	+	++	+	++	++	++	++
	Spore	++	++	-	++	-	++	-	-	-	-
A.fumigatus	Growth	-	-	-	+	-	-	-	-	+	-
	Spore	++	++	++	++	++	++	++	++	++	++
P. chrysogenum	Growth	++	++	++	++	++	-	++	++	++	++
	Spore	-	-	-	-	-	-	-	++	++	-
A. oryzae	Growth	++	++	++	++	++	++	++	++	++	++
	Spore	-	++	-	++	++	-	-	-	-	-
A. alternata	Growth	+	++	+++	-	-	++	+++	++	+++	++
	Spore	++	++	++	++	++	-	++	-	++	-

(-)No inhibition, (+) weak inhibition, (++) inhibition, (+++) strongly inhibition

Table 3: Inhibition of conidia and mycelia growth in the well methods.

11 was reported to inhibit the growth of *Fusarium culmorum* and *P. expansum* [14].

Changes in *P.chrysogenum, P. griseofulvum and E. durans* counts during the six days in MRS broth are given Figure 2. The population of *P.chrysogenum, P. griseofulvum,* in the inoculated samples decreased gradually during the six days. The count of *P.chrysogenum* decreased below the minimum detectable level after 48 h. There for *P.chrysogenum* was not detected in the medium. *P.chrysogenum* was more sensitive to metabolites of *E. durans. P. griseofulvum* exhibited significant tolerance to the medium after 6 days. The count of *P. griseofulvum* decreased below the minimum detectable level after 6 days (Figure 2).

Figure 3 shows the survival of *A. fumigatus and A. parasiticus* in the MRS broth. The population of *A. fumigatus and A. parasiticus* decreased gradually during the first four days. The count of *A. parasiticus* was below the minimum detectable level after 6 days. *A. fumigatus* was not inhibited (Figure 3). Wiseman and Marth [15] demonstrated inhibition of *A. parasiticus* by *Streptococcus lactis* C10 strain, without identification of the inhibitor agent.

Finally, *E. durans* completely inhibited the growth of filamentous fungus *P.chrysogenum* after 48 h. *P.griseofulvum* and *A. parasiticus* inhibited after 6 days.

P.chrysogenum, P. griseofulvum, A. parasiticus were almost

Growth At	Growth In
F	
+	Gram reaction
co	Morphology
-	Alpha hemolysis
-	Beta hemolysis
+	Voges-Proskauer
-	Catalase
-	H_2S production
+	Arginine
-	4ºC
+	15ºC
+	45ºC
+	6.5% NaCl
+	7.0% NaCl
-	10% NaCl
-	pH 2
-	pH 3.9
+	pH 9.6

Table 4: Morphological, cultural, and physiological characteristics of the F 2.1.

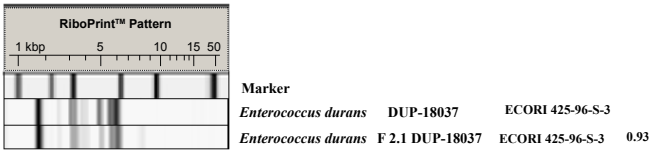

Figure 1: Ribotyping profiles of isolate and standards were shown with DUP numbers.

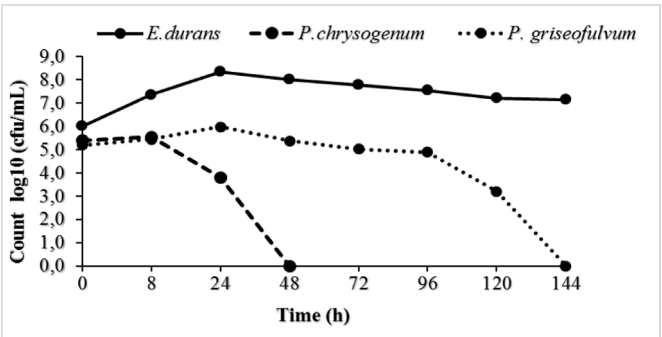

Figure 2: Inhibition of *P.chrysogenum* and *P. griseofulvum* by *E. durans* F2.1 in MRS broth.

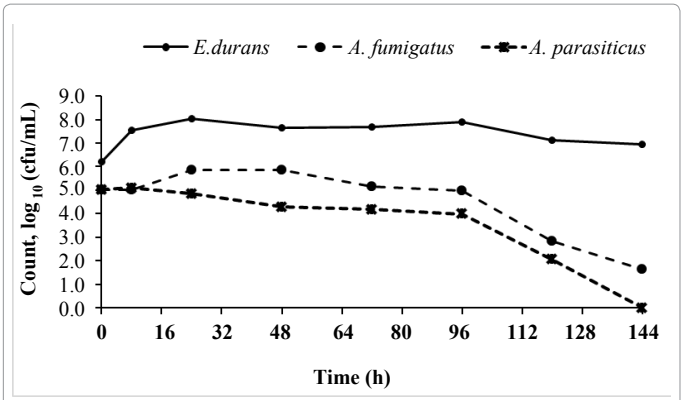

Figure 3: Inhibition of *A. fumigatus and A. parasiticus*. by E. *durans* F2.1 in MRS broth.

completely inhibited by *E. durans. A. fumigatus* was shown to be resistant to the *E. durans*. The inhibition of fungal growth showed high sensitivity to evaluate inhibition on all fungal strains tested except *A. fumigatus*.

This is the first report on the antifungal performances of *E. durans* against *P.chrysogenum, P. griseofulvum, A. parasiticus.*

The inhibitory activity of *E. durans* against tested fungi (*P.chrysogenum, P. griseofulvum, A. parasiticus)* could be production of proteinaceous substances and organic acids. Belguesmia et al. [14] reported that the antifungal activity of *E. durans* have 2 bacteriocins named duracin. Laitila et al. [16] and Lavermicocca et al. [17] suggested that the antifungal activity of *L. plantarum* could be the results of many organic acids such as lactic, acetic and phenyllactic acids.

More than one compound is responsible for the antimicrobial activity of *E.durans*. We need further studies to use these compounds alone or in combination for their potential use as food biopreservation. *E.durans* can be used as starter culture in foods. With this method itself of *E.durans* can be biopreservation directly, however we also need further studies about *E.durans*.

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