Screening of Lactic Acid Bacteria Isolated from Iranian sourdoughs for Antifungal Activity: *Enterococcus faecium* showed the Most Potent Antifungal Activity in Bread

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Abstract

Background and Objective: The use of antifungal lactic acid bacteria as starter for bread making could be a good alternative to improve the stability of bread shelf life.

Material and Methods: In this study, a total of 57 lactic acid bacteria were isolated from spontaneously fermented wheat sourdoughs collected in Chahar-Mahalo Bakhryari province of Iran. The isolates were screened for in vitro antifungal activity (towards *Aspergilus niger* or *Penicillium roqueforti*); and the selected isolates (six isolates) were applied in flat bread making. The freshly baked breads were nebulized with a suspension of either molds, containing 10^4 spores ml^{-1}, and the fungal growth on breads was monitored over a 7-day storage period.

Results and Conclusion: Bread produced with either isolates AN3 and MB1 (both were identified as *Enterococcus faecium*) restrained the growth of *Aspergillus niger* for up to 5 days. Even though none of the isolates were strong enough to inhibit the growth of *Penicillium roquforti* on bread, the surface area of breads contaminated by this fungus was significantly lower than the control samples. To our knowledge, it was the first report indicating the anti-mold activity of *Enterococcus faecium* strains isolated from sourdough. These isolates seem to be promising for further analysis and their application in bread industry for prolonging the shelf life.

Conflict of interest: The authors declare that there is no conflict of interest.

1. Introduction

Fungal spoilage leads to about 20% of economic losses in bakery industries as well as reducing the safety for consumers due to the production of mycotoxins [1,2]. Nowadays, the consumers are looking and demanding for products without chemical preservatives and, still maintaining good shelf life and safety [3]. In the last few years, the use of microorganisms and/or their metabolites to prevent spoilage of food products has gained the interest of food producers in different sectors [4-6]. This application has been called bio-preservation. The bioactive metabolites that contribute to product preservation can be simply organic acids, fatty acids, and hydrogen peroxide, or more specific components such as bacteriocins, which are produced by some strains during the growth period [7].

Sourdough is the oldest bread production technique, which is still in use in some regions as a way of bread making. The natural microflora of sourdough includes yeasts and lactic acid bacteria (LAB), which are active in

the leavening of the dough, alcohol fermentation, and in the souring of the dough, [8].

Spontaneous and/or traditional fermented products are rich sources of autochthonous lactic acid bacteria that can be applied in different research studies in order to characterize them for special functionality and applications [9-11]. The sourdough ecosystem has been proved to be very diverse concerning its LAB [12]. *Lactobacillus (L.) sanfranciscensis, L. plantarum, L. rossiae,* and *Weissella (W.) cibaria* are considered as highly adapted to this niche and recognized as the key sourdough microorganisms [12]. Some of these strains have shown antifungal activities via both in vitro and in vivo experiments. *Penicicllium (P.) roqueforti* and *Aspergillus (A.) niger* will be used as fungal indicator in the current study. *Penicillium* spp. cause around 90% of wheat bread spoilage [13] and *P. roqueforti* has indicated as the most resistant fungus to antifungal compounds in many studies [1,14]. Ryan et al. [1] were the first authors to report an inhibitory activity against *P.*

roqueforti in an in vivo study and it was upon applying *L. plantarum* fermented sourdough in bread production. *A. niger* is able to grow even in low temperature and at pH 2.5, Gerez et al. [15] considered this fungus as the most resistant through analysis of anifungal activity of 91 LAB.

Although, a large number of studies have done on extensive analysis of the microbial composition of sourdoughs from various european countries during the last years [11,12,16-18], not enough studies have been done on samples originated from non-European countries. As the geographic properties, type of flour and the concentration of some flour nutrients required by the microorganisms have a key role for dominating of specific population of LAB [17]. It is worthwhile to study the sourdoughs from less studied regions. In Iran, sourdough is still being used in some rural regions for bread backing [19], however, just a limited number of studies [19-21] have been carried out on investigation and characterization of microbiota of these traditional sourdoughs. In Charmahalo-Bakhtiyari region owning to its traditional culture, still many habitants are using spontaneous sourdoughs in bread preparation process. The current study aimed to isolate the pre-dominant LAB from a number of sourdough collected from this region and evaluate their antifungal performance with the objective of applying the selected isolates in flat bread production in order to prolong bread's shelf life.

2. Materials and Methods

2.1 Sampling and isolation

Twenty-one samples of homemade spontaneously fermented wheat sourdough from the region of Chaharmahalo-Bakhtiyari, Iran, have been collected and used in this study. After transformation to the laboratory, pH (WTW, England) was recorded, and samples (10 g) were homogenized with 90 ml of 0.9% NaCl using Stomacher apparatus. Numbers of colony-forming units were determined by plating serial dilutions on Yeast extract-Glucose- Chloramphenicol (YGC) agar (Biolife, Italy) for yeast and on modified DeMan-Rogosa-Sharpe (mMRS) agar (Biolife, Italy) supplemented with 2% (w v^{-1}) maltose (Biolife, Italy). YGC and mMRS agar plates were incubated at 25 and 37°C for 48 h respectively, and afterwards they were used for enumeration of colonies. In order to isolate lactic acid bacteria, 3-4 colonies were picked up from the final dilution of each sample grew on mMRS agar, analyzed for catalase activities and purified by successive streaking on MRS agar for 3 times. Isolates were stored at -20°C in MRS broth, containing 25% (v v^{-1}) glycerol.

A. niger IBRC-M-30064 and *P. roqueforti* IBRC-M-30025 (all obtained from the culture collection of the Iranian Biological resource Center (IBRC), Iran) was used as the indicator fungi in the antifungal activity assay.

They were grown on YPG agar (consisting of yeast extract 3 g l^{-1}, peptone 15 g l^{-1}, glucose 5 g l^{-1}, agar 15 g l^{-1}) until sporulation occurred. Fungal conidia were harvested from the plates in physiological solution containing 1% (v v^{-1}) Tween 80 and the concentration of conidia was determined by using a hemocytometer. For long pre-servation, harvested spores were stored in a glycerol:water (50:50 % v v^{-1}) solution at -80°C.

2.2. Evaluation of in vitro antifungal activity

Two different assays including the overlay and well diffusion methods were employed to determine the in vitro antifungal activity of the isolates, assays adapted and performed according to Corsetti et al. [22]. Inhibitory activity was first evaluated with the overlay method and the isolates displayed antifungal properties were further subjected to the well diffusion assay after preparation of their cell free supernatant (CFS, centrifugation at 4,000 ×g for 10 min in sterile condition).

In overlay method, 6 µl of each overnight grown culture (in MRS broth at 30°C) with the concentration of 10^8 colony-forming units (CFU) ml^{-1} were spotted on MRS agar. The plates were incubated at 32°C for 48 h in anaerobic jar. The plates were overlaid with soft YPG agar (consisting of agar 7.5 g l^{-1}) before inoculation with each indicator (final concentration of 1×10^5 spores ml^{-1}). The plates were incubated at room temperature for 3 days and the inhibitory activity was scored as follows: (-) no inhibition, (+∕-) spore formation delayed but no clear zone (this was evaluated as fungistatic activity), (+) a very good inhibition activity against mycelium and conidia growth with large than 1 mm clear zones around colony (this was evaluated as fungicidal activity).

2.3. Molecular identification of isolates

Identification of the selected isolates was carried out by 16S rDNA gene sequencing using the primer oligo-nucleotides fD1 (5`-AGA GTT TGA TCC TGG CTC AG-3`) and rD1 (5`-TAA GGA GGT GAT CCA GC-3`) [23] provided by Eurofins, Germany. DNA amplification were carried out in 100 µl reaction mixtures containing 20 µl of 5 x PCR buffer, 2 µl dNTP mixture (20 mM), 1.5 µl MgCl2 (50 mM), 1 µl of each primers (100 pmol µl^{-1}), 1 µl of DNA (5-10 ng) and 1 µl Q5® High-Fidelity DNA Polymerase (2 U µl^{-1}). The polymerase chain reaction condition was 1 cycle at 95°C for 2 min; 95°C for 40 s, 55°C for 40 s, and 72°C for 60 s (30 cycles) following a final extension at 72°C for 5 min. The amplified fragments were purified and sequenced by the GATC Biotech (Koln, Germany).The resulting sequence was used to search the sequence for the 16 rDNA using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [24].

2.4. Sourdough preparation for bread making

100 ml of MRS broth was inoculated by each freshly grown isolates and incubated at 32°C for 20 h. Cells were harvested by centrifugation (2500 ×g, 10 min), washed once and resuspended in sterile tap water to an OD_{600} 1.0 that is equal to 1×10^9 CFU ml^{-1}. Each bacterial suspension was added to the sourdough to an initial inoculum size of 1×10^8 CFU g^{-1} dough. Sourdoughs were prepared with dough yield (DY) of 180, and were fermented at 32°C for 19 h. Total titrable acidity (TTA) and pH values of the fermented sourdoughs were measured.

To conduct the in situ shelf life test, sourdough samples were incorporated into a simple flat bread recipe (20% w w^{-1} flour-based). A control sample (SCC) containing commercial baker's yeast was prepared to compare the inhibitory activity of the sourdoughs to that of Saccharomyces (S.) cerevisiae. In addition, a chemically acidified sample (by addition the mixture of lactic and acetic acid (4:1, v v^{-1}), without incubation, was prepared and considered as chemically acidified control (CAC).

All prepared dough samples were incubated at 32°C for 5h and then backed. After baking, the breads were cooled at ambient temperature and then sprayed with each suspension of *A. niger* IBRC-M-30064 and *P. roqueforti* IBRC-M-30025 (10^5 conidia ml^{-1}) separately. Breads were packed with polyethylene film individually and stored at room temperature. The fungal contamination of the bread slices was monitored over a period of 7 days. Mold growth was evaluated based on the percentage of the total surface area of each bread where outgrowth of fungi occurred [4].

2.5. Statistical analysis

GraphPad Prism version 6.0 (GraphPad Software, La Jolla, Canada) was used for analysis of data, pH and TTA for sourdough and bread samples. Results were expressed as the mean and standard error and the differences between means were evaluated by Tukey's test. Data were considered significantly different when the p-values were ≤ 0.05. Preparation of sourdough and breads were performed in 3 replicates.

3. Results and Discussion

In this study, 21 samples of homemade spontaneously-fermented sourdoughs from the region of Chaharmahalo-Bakhtiyari, Iran, have been applied in LAB isolation. The pH and microbial counting of these samples are shown in Table, 1 pH value of sourdoughs ranged from 3.3 to 5.4. The population of LAB and yeast in these samples ranged from 2.50×10^7 to 8.50×10^8, and 1.21×10^5 to 1.00×10^7 CFU g^{-1}, respectively. Although Ottogalli et al. concluded that yeasts LAB ratio is generally 1:100, many studies have shown that this ratio can be very diverse according to several intrinsic and environmental parameters, such as pH, water content, time and temperature of incubation during sourdough production [25,26,27]. In addition, interaction of LAB and yeast on each other (for example production of inhibitory products) has been considered as an important factor affecting the numbers in each group [27]. However, our results are in agreement with Lattanazi et al. and *Minervini* et al. that reported the number of LAB and yeasts in sourdough ranging from 1.00×10^6 to 1.00×10^9 CFU g^{-1} and from 1.00×10^6 to 1.00×10^9 CFU g^{-1}, respectively.

In total 57 predominant LAB strains were isolated from sourdough samples in the current study and all strains were applied in the antifungal activity.

Table 1. pH and microbial count of sourdough samples used for isolation of predominant LAB

No.	Names was given to samples	Sampling location	pH	Yeast*	MRS*
1	EL	Farsan	3.3	7.00×10^5	1.10×10^8
2	AN	Boroojen	4.4	4.00×10^6	1.75×10^8
3	GR	Boldaji	5.4	5.60×10^5	6.92×10^8
4	GB	Boldaji	5.0	3.80×10^6	7.60×10^8
5	SZ	Gandoman	3.9	1.30×10^6	7.50×10^8
6	DA	Gandoman	4.0	4.40×10^6	2.95×10^8
7	KR	Boroojen	4.7	3.60×10^5	2.50×10^7
8	ZF	Gandoman	4.5	1.60×10^6	5.60×10^8
9	ZA	Gandoman	4.7	8.60×10^5	4.00×10^8
10	MB	Lordegan	4.5	4.30×10^6	6.40×10^8
11	AV	Gandoman	4.6	3.60×10^5	3.00×10^8
12	TS	Shahrekord	4.0	9.10×10^5	4.30×10^7
13	AF	Boroojen	4.5	3.40×10^6	3.80×10^8
14	KN	Shahrekord	4.4	1.00×10^7	5.00×10^8
15	AB	Koohrang	4.7	7.00×10^6	6.00×10^8
16	AA	Koohrang	5.2	1.61×10^6	4.00×10^8
17	ZT	Gandoman	4.7	9.00×10^5	1.20×10^8
18	TD	Shahrekord	4.5	1.21×10^5	1.80×10^8
19	KH	Shahrekord	5.2	1.50×10^6	3.00×10^7
20	TL	Lordegan	4.7	6.11×10^6	2.00×10^8
21	TB	Boroojen	4.3	7.00×10^6	8.50×10^8

* Population are in CFU g^{-1}

3.1 Antifungal activity

The overlay method was adopted in order to investigate the inhibitory activity of 57 LAB isolates towards 2 fungal indicators. As indicated in Table 2, 6 isolates out of 57 showed antifungal activity towards at least one of the 2 fungal indicators. According to the results isolates EL2, AN3, GR2, SZ5, DA1, MB1 showed antifungal activity towards *A. niger*, and isolates EL2, AN3, SZ5, MB1 towards *P. roqueforti*. Isolates EL2, AN3, SZ5 and MB1 have marked activity towards both spoilage indicators (Table 2). According to our results, *P. roqueforti* was more resistant than *A. niger* and none of the isolates could not inhibit its growth on agar plate and just its sporulation. In case of *A. niger,* 4 isolates (AN3, GR2, SZ5, MB1 and DA1) inhibited this fungus with clear zone near the rim of the colony (ca. 4.9 ± 0.3, 8.1 ± 0.5, 5.2 ± 0.4, 1.8 ± 0.3 and 3.7 ± 0.2 mm, respectively).

Table 2 shows the results of identification according to 16s rDNA sequencing. According to this, the isolates that inhibited *A. niger*, with the highest observable clear zone of inhibition, were identified as *W. cibaria*, *L. plantarum, Enterococcus (E.) faecium* and *Leuconostoc (Leuc.) mesenteroides* subsp. *mesenteroides*, respectively. The isolates that inhibited sporulation of *P. reoqueforti* were identified as *L. pentosus, L. plantarum* and *E. facieum*.

Different species of Enterococci, including *E. faecium*, have been frequently isolated from wheat grain, flour and sourdough samples [11,18]. Corsetti et al. [18] reported *E. faecium* as one of the most frequently found species that is not generally reported to be typical in mature sourdoughs, as they are unable to survive a long-term acidification. According to these authors, *E. faecium* plays a crucial role at the beginning of fermentation due to their ability to degrade maltose and accumulating glucose, which may be then utilized by maltose-negative strains [18]. However, reports on antifungal prowess of enterococci, including *E. faecium* are relatively rare [28,29], and to the best of our knowledge, no study has reported the anti-mold activity of *E. faeciam* strains isolated from sourdoughs. Just recently, Roy et al. characterized the anti-candida activity of a bacterocin substance synthetize by a *E. faecium* strains isolated from a penguin rookery of the Antarctic region [28]. Belguesmia et al. reported antifungal activity of a strain *E. durans*, was isolated from Mongolian airag cheese, towards *P. roqueforti* by delaying growth of this fungus [30]. Ohhira et al. reported the production of phenyllactic acid by a *E. faecalis* strain isolated from traditional fermented tempeh [31]. This substance has approved for its antifungal activity [32] and has been identified in supernatant of many LABs with antifungal activity like *L. plantarum* strains FST 1.7 and 21B [33,34].

3.2. Sourdough and bread manufacture

All six isolates that showed antifungal activity were applied in the form of sourdough in bread dough preparation. The final pH and TTA values in all sourdough samples were from 3.6 ± 0.2 to 4.2 ± 0.1, and $10.3 \pm 0.4\%$ to $6.1 \pm 0.2\%$, respectively, following 20 hour fermentation. The lowest pH and the highest TTA were observed in the sourdough sample fermented with isolate SZ5 (was identified as *L. plantarum*), however, it was not significantly ($p \leq 0.05$) different from values recorded for sourdough sample prepared with isolate AN3 (was identified as *E. faecium*). Sourdough samples prepared with either isolates GR2 and DA1 showed significantly ($p \leq 0.05$) higher pH and lower TTA after 20 h fermentation compared to other samples. This was predictable as these two isolates were identified as *W. cibaria* and *Leuc. mesenteroides*, respectively, which both are classified as obligate heterofermentative LAB [29]. In all samples, cell counts reached concentrations between 8.80×10^8 and 3.20×10^9 CFU g^{-1} after 20 h of fermentation (data were not shown).

Table 2. Inhibitory activity of LAB isolates towards *A. niger* and *P. roqueforti* and identification of isolates by 16s rDNA sequencing

Isolate name[a]	Antifungal activity [b]		Closest relative	Identity, %	Diff/Tot nt	Accession No.
	A. niger	*P. roqueforti*				
EL2	+/-	+/-	*L. pentosus*	100.0	0/794	D79211
AN3	+	+/-	*E. faecium*	99.90	1/1025	AJKH01000109
GR2	+	-	*W. cibaria*	100.0	0/947	AEKT01000037
SZ5	+	+/-	*L. plantarum*	100.0	0/840	ACGZ01000098
DA1	+	-	*Leuc. mesenteroides subsp. Mesenteroides*	99.89	1/927	CP000414
MB1	+	+/-	*E. faecium*	99.79	2/953	AJKH01000109

[a] The name of isolate come from the name of sourdough that the isolate has been isolated from

[b] (-) no inhibition, (+/-) spore formation delayed but no clear zone, (+) a very good growth inhibition with larger than 1 mm clear zones around colony.

Table 3. Recipes of the sourdough based dough (containing 20% of sourdough on the basis of flour) and two controls

	SCC	CAC	Sourdough-based dough
Wheat flour	100	100	80
Sourdough	-	-	20
Water	75	75	75
Salt	1	1	1
Yeast	1	-	-
Acid mix[a]	-	1.25	-

[a] SCC: Fermented by *Saccharomyces cerevisiae*

[b] CAC: Chemically acidified control adjusted using mixture of lactic and acetic acid (4:1, v v^{-1})

Sourdough samples of previous steps were used in bread dough preparation. Six sourdough-based breads and two control breads (*S. cerevisiae*-fermented or artificial acidified breads) were manufactured at pilot plant scale. In this step, after 5 h fermentation, pH values of bread dough samples, even having slight differences (from 4.5 ± 0.2 to 4.8 ± 0.1) were not significantly different ($p \leq 0.05$), although significant differences ($p \leq 0.05$) were observed among samples regarding TTA (from $4.0 \pm 0.3\%$ to $5.0 \pm 0.4\%$) (Table 4). The pH and TTA values in the CAC (chemically acidified control) were comparable with the lowest pH 4.5 ± 0.2 recorded for sourdough-fermented dough, fermented with isolate SZ5 (which was identified as *L. plantarum*). As expected, the dough prepared with commercial *S. cerevisiae* had the highest pH and overall lowest TTA values in comparison to fermented dough breads by LAB ($p \leq 0.05$). All in all, the pH and TTA values

of sourdough and bread dough samples observed in this study are in accordance with the previous studies [35-37].

The study on bread spoilage using severe mold environmental challenge method (nebulizing a suspension containing 10^5 conidia ml^{-1} of fungi spores on freshly baked bread) was conducted to assess the in situ antifungal activity of the selected LAB isolates. Table 5 represents an overview of the shelf life for the sourdough-based breads as well as for the control samples over a period of 7 days. As determined by a visual inspection, after 2 days of baking the fungal contamination (especially contamination by *P. roqueforti*) was clearly visible in both controls (non-acidified and chemically acidified), however, no fungal outgrowth was detected on sourdough-based breads. During the following days, fungi grew more rapidly on both controls, whereas fungal outgrowth could partially prevent in sourdough-based breads. At 5th day of monitoring, contamination by *P. roqueforti* was observable on the surface of all sourdough-based breads, and the breads prepared by isolates GR2 and EL2 (*W. cibaria* and *L. pentosus*, respectively, according to 16s rDNA identification) showed the highest and the lowest percentage of surface contamination respectively. Regarding to spoilage by *A. niger*, no spoilage were observed on breads prepared with sourdough fermented by each isolates AN3 and MB1 (both were identified as *E. faecium*). At 5th day, *A. niger* covered more than 80% of breads manufactured by commercial *S. cerevisiae*. As contamination of CAC bread was comparable to sourdough-breads, we concluded that acidification by organic acids can be a strong factor in retarding the growth of both fungi during the first 5 days.

Table 4. pH and TTA values of sourdough samples (20 h of fermentation) and sourdough-based dough (5 h of fermentation, before backing) fermented by SZ5, AN3, GR2, EL2, MB1 and DA1, as well as dough prepared by *S. cerevisiae* (SCC)

Sample ID	Sourdough[a]		Bread dough[a]	
	pH$_{20}$	TTA$_{20}$[b]	pH$_5$	TTA$_5$
SZ5	3.6 ± 0.2^a	10.3 ± 0.4^c	4.5 ± 0.2^a	4.8 ± 0.3^c
AN3	3.9 ± 0.2^a	9.2 ± 0.2^c	4.6 ± 0.2^a	4.2 ± 0.3^b
GR2	4.2 ± 0.1^b	6.8 ± 0.2^a	4.7 ± 0.2^a	4.0 ± 0.2^b
EL2	4.0 ± 0.1^b	10.2 ± 0.3^c	4.5 ± 0.2^a	5.0 ± 0.4^c
MB1	4.1 ± 0.2^b	8.2 ± 0.2^b	4.7 ± 0.1^a	4.1 ± 0.3^b
DA1	4.2 ± 0.1^b	6.5 ± 0.3^a	4.8 ± 0.2^a	4.0 ± 0.2^b
SCC[c]	-	-	5.9 ± 0.1^b	2.8 ± 0.3^a

[a] The fermentation time for sourdough and bread dough were 20 and 5 h, respectively (refer to material and methods)

[b] TTA is percent of lactic acid

[c] SCC: Fermented by *Saccharomyces cerevisiae*

Mean values ± SE, n = 3

Different letters (a-d) show statistical differences between strains, in column ($p \leq 0.05$)

At the end of our monitoring (7th day), both controls (SCC and CAC) were completely spoiled by both fungal indicators. MB1 and AN3-fermented sourdough breads (both were identified as *E. faecium*), both were able to limit the contamination by *A. niger* to about 10% of bread surface, which followed by GR2 and DA1-fermented sourdough breads (were identified as *W. cibaria* and *Leuc. mesenteroides* subsp. *mesenteroides*, respectively). However, *P. roqueforti* showed lower susceptibility to antifungal compounds and could cover the surface of sourdough-based breads by two times more than *A. niger*. Table 5

An in situ antifungal activity confirmation in bread is normally necessary for those isolates that already have shown activity on plate, due to this fact, backing conditions can cause losses in concentration of antifungal compounds [4]. Digaitiene et al. [38] revealed that the resistance of bacteriocin-like substances to heating depends on the producer strain. These authors, for instance, showed that pediocin Ac05-7 appeared to be stable even after treatment at 100°C for 60 min, though the activity of pediocin 05-9 and pediocin 05-10 was totally lost after treatment at 100°C for 30 min. In our case, sourdough-based bread showed remarkable retarding of fungal growth at least through the first 5 days of storage. However, the main reason of early and fairly quick fungal growth on the surface of breads in our study could be as a result of nebulization of a high concentration of spores (10^5 spores ml^{-1}) on breads which is about 10 times higher than the concentrations that have been used in the similar experiments in some other studies [1,39].

According to the findings of current study, the antifungal activity of isolate GR2, identified as *W. cibaria,* towards *A. niger* was more effective than *L. plantarum*

SZ5 and *L. pentosus* EL2 in both analysis of spot-agar and bread system. *W. cibaria* is an obligately hetero-fermentative LAB, that has been isolated as a dominant LAB from different sources of sourdough [40]. Leuco-nostoc and Weissella spp. may play a role during the first phase of the fermentation and they can be important for growth association with lactobacilli [41]. Even though many probiotic and technological properties of Weissella genus have been recently reported [7,40], very few information has been reported about their antifungal activity [7,16,42]. Our results confirm the observation of Valerio et al. who also measured a significantly stronger antifungal activity for *W. cibaria* sp. than *L. plantarum* sp. [16]. Beak et al. successfully applied an antifungal strain of *W. confuse*, a closely related species to *W. cibaria*, in rice cake formulation in order to prolong the shelf life of the product [7].

L. plantarum was another isolate that showed retarding effects on bread molds growth. *L. plantarum* is a facultative heterofermentative and has been frequently isolated as a main LAB from type 1 sourdoughs in many studies that highlights the relevance of this species to produce sourdoughs throughout the world [8,43-44]. *L. plantarum* is well known for producing bacteriocin (plantaricin) with antifungal activity, albeit the production of antimicrobial compounds is not a general characteristic in all *L. planatum* strains. Although, several strains of *L. plantarum* have been reported for their notable antifungal activity against common bread contaminants [13]; the activity seems to be very strains-dependent. For example, Dal Bello et al. showed that *L. plantarum* strain FST 1.7 is a potent antifungal strain against *A. niger* and 4 strains of Fusarium, but showed no activity against *P. roqueforti* [5], that is in accordance with our results regarding isolate SZ5.

Table 5. Outgrowth of *A. niger* and *P. roqueforti* on sourdough-based breads as well as control breads over a period of 7 days storage

Storage time (days)	Contamination of bread samples [a]							
	Spraying of *A. niger*							
	SZ5	AN3	GR2	EL2	MB1	DA1	SCC[b]	CAC[c]
2	-	-	-	-	-	-	+	+/-
5	+	-	+/-	+	-	+/-	+++	+
7	++	+/-	+	++	+/-	+	++++	+++
	Spraying of *P. roqueforti*							
	SZ5	AN3	GR2	EL2	MB1	DA1	SCC[b]	CAC[c]
2	-	-	-	-	-	-	+/-	+
5	+	+	++	+/-	+	++	++	++
7	++	++	+++	++	++	+++	++++	++++

[a] Contamination was scored as follows: -, 0% contamination of the bread surface; +/-, 10% contamination; +, 20% contamination; ++, 40% contamination; +++, 80% contamination, ++++, 100% contamination. For each bread the surface was inoculated by spraying a suspension of 10^5 conidia ml^{-1} of *A. niger* IBRC-M-30064 and *P. roqueforti* IBRC-M-30025.

[b] SCC: Fermented by *Saccharomyces cerevisiae*

[c] CAC: Chemically acidified control

4. Conclusion

This was the first report of antifungal performance of *E. faecium* strains isolated from sourdough against fungal species of spoilage in bakery products. The breads manufactured with sourdoughs fermented by either *E. faecium* AN3 or MB1 achieved a *A. niger*-free shelf life of 5 days, even though a high concentration of spores (10^4 spores ml^{-1}) had been sprayed on the surface of breads after baking. These findings reveal that *E. faecium* can be a good candidate for naturally retarding of the outgrowth of environmental fungi. However, more research is required to identify the component(s) that are responsible for this activity. In agreement of the previous research, data from this study confirms the significance of the exploration of different ecological niches to highlight the antifungal performances of LAB.

5. Acknowledgements

6. Conflict of Interest

The authors declare that they have no conflict of interest.

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غربالگری باکتری‌های اسید لاکتیک جدا شده از خمیرترش‌های سنتی ایران به منظور بررسی فعالیت ضدقارچی آن: *انتروکوکوس فیسیوم* قوی‌ترین فعالیت ضد قارچی در نان

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چکیده

سابقه و هدف: در تولید نان، استفاده از باکتری‌های لاکتیک اسید دارای فعالیت ضدقارچی، به عنوان آغازگر، می‌تواند جایگزینی خوب برای بهبود پایداری و افزایش عمر نگهداری نان باشد.

مواد و روش‌ها: در این مطالعه ۵۷ باکتری لاکتیک اسید از خمیرترش‌های حاصل از تخمیر خود به خود گندم، جمع آوری شده از استان چهارمحال و بختیاری ایران، جداسازی شد. این جدایه‌ها براساس توانایی ضد قارچی در برابر کپک‌های آسپرژیلوس نایجر یا پنی سیلیوم *راکفورتی* در شرایط آزمایشگاهی غربالگری شدند. از جدایه‌های انتخابی (شش جدایه) در تولید نان مسطح مورد استفاده قرار گرفتند. نان‌های تازه پخته شده با سوسپانسیونی از هریک از کپک‌ها با غلظت ۱×۱۰۴ CFU ml^{-1} اسپری شدند و رشد قارچی بر روی نان‌ها طی دوره ۷ روزه نگهداری پایش شد.

نتایج و بحث: تا ۵ روز، رشد آسپرژیلوس نایجر در نان تولید شده با هریک از جدایه‌های AN3 و MB1 (که هر دو به عنوان *انتروکوکوس فیسیوم* شناسایی شدند) مهار شد. اگرچه هیچ یک از این جدایه‌ها نتوانستند مانع از رشد کپک پنی‌سیلیوم *راکفورتی* بر روی سطح نان شوند؛ میزان آلودگی سطح نان آلوده به این کپک، به میزان قابل توجهی کمتر از نمونه‌های شاهد بود. تا جایی که می دانیم، تحقیق حاضر اولین گزارشی است که بر فعالیت ضد قارچی سویه *انتروکوکوس فیسیوم* جدا شده از خمیرترش را نشان داده است. به نظر می‌رسد تحلیل بعدی و کاربرد این جدایه‌ها در صنعت نان، به منظور افزایش عمر انباری، امیدوار کننده باشند.

تعارض منافع: نویسندگان اعلام می کنند که هیچ تعارض منافعی وجود ندارد.