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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Lactic acid bacteria as functional probiotic isolates for inhibiting the growth of *Aspergillus flavus*, *A. parasiticus*, *A. niger* and *Penicillium chrysogenum*

*Les bactéries lactiques en tant qu'agents probiotiques pour l'inhibition de la croissance d'*Aspergillus flavus, A. parasiticus, A. niger *et de* Penicillium chrysogenum

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KEY-WORDS
Antifungal activity;
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Aspergillus;
Penicillium

Summary
Objective. — The aim of this study was to assess the potential of lactic acid bacteria (LAB) such as *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casei*, *L. paracasei* and *Bifidobacterium bifidum* to inhibit the outgrowth of some common food-spoiling fungi including *Aspergillus niger*, *A. flavus*, *A. parasiticus* and *Penicillium chrysogenum*.
Methods. — Bacterial isolates were cultured on Mann Rogosa Sharpe (MRS) broth and liquid cultures and supernatants were prepared. The antifungal activity was tested using the agar well diffusion method.
Results. — Both liquid culture and supernatant of *L. casei* isolate exhibited high antifungal activity, followed by *L. acidophilus* and *L. paracasei* isolates. The least activity was recorded for the isolates *B. bifidum*, while the isolate *L. rhamnosus* was moderately active against tested fungi. The antifungal activity of the supernatants obtained from all probiotic isolates against fungi was significantly less than that of liquid cultures ($P < 0.05$). Antifungal activity evaluation

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showed that *A. flavus* was the most inhibited fungus by probiotic bacteria, followed by *P. chrysogenum*, *A. niger* and *A. parasiticus*.
Conclusion. — These results suggest that probiotic bacteria strains have the ability to prevent the growth of pathogenic and mycotoxigenic fungi as antifungal agents for various biomedical applications.
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MOTS-CLÉS
Activité antifongique;
Probiotic;
Lactobacillus;
Bifidobacterium;
Aspergillus;
Penicillium

Résumé
Objectif. — Le but de cette étude était d'évaluer le potentiel de bactéries lactiques comme *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casei*, *L. paracasei* et *Bifidobacterium bifidum* pour inhiber la croissance de quelques champignons communs altérant la nourriture comme *Aspergillus niger*, *A. flavus*, *A. parasiticus* et *Penicillium chrysogenum*.
Matériel et méthodes. — Les isolats bactériens ont été cultivés sur bouillon de Mann Rogosa Sharpe (MME) et les liquides de culture et les surnageant ont été préparés. L'activité antifongique a été évaluée en utilisant la méthode de diffusion en puits de gélose.
Résultats. — La culture liquide et le superrnageant de *L. casei* ont montré la plus forte activité antifongique suivie par *L. acidophilus* et *L. paracasei*, mais les différences entre les deux étaient non significatives ($p < 0{,}05$). La plus faible activité a été enregistrée avec *B. bifidum*, alors que *L. rhamnosus* était modérément actif contre les champignons testés. L'évaluation de l'activité antifongique a montré que *A. flavus* était le champignon le plus inhibé par les bactéries probiotiques, suivi par *P. chrysogenum*, *A. niger* et *A. parasiticus*.
Conclusion. — Ces résultats suggèrent que les bactéries probiotiques ont la capacité d'empêcher la croissance de champignons pathogènes et mycotoxicogènes et sont des agents antifongiques potentiels pour les applications biomédicales.
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Introduction

A wide spectrum of filamentous fungi and yeasts is often found in various food commodities, where they can cause extensive damage and lead to sizable economic losses [19]. Fungal infection leads to food spoilage such as off-flavors, discoloration, rotting and disintegration of the food structure [2,30]. The very important aspect involved in spoilage of food by fungi is also the formation of toxic secondary metabolites — mycotoxins. Concerning the importance and diversity of their toxic effects including carcinogenic, teratogenic, mutagenic, immunotoxic, neurotoxic, nephrotoxic and hepatotoxic properties, the occurrence of mycotoxigenic fungi in foods constitutes a high risk for human and animal health [28]. Although prevention of fungal growth and mycotoxin production on plants and in feedstuffs is usually considered as the best approach to impede the harmful effects on animal and human health, decontamination/detoxification of contaminated products is also of prime importance [33]. Several physical and chemical compounds are used for the preservation of food and feed. However, some filamentous fungi and yeasts have acquired the ability to resist chemical treatments and some preservatives [21]. There is a great risk that the resistance phenomenon will increase in the future due to the frequent use of antibiotic and preservatives.

Biopreservation, the control of one organism by another, could be an interesting alternative to physical and chemical methods, and it has received much attention lately [18]. Among the different potential decontaminating microorganisms, the group of lactic acid bacteria has been considered as the most promising natural biological antagonists. Lactic acid bacteria (LAB) are a group of gram-positive, non-spore forming cocci or rods, which produce lactic acid as a major end product from fermentation of carbohydrates [24]. The majority of microorganisms used as probiotics belong to LAB and bifidobacteria [10]. The selection criteria for probiotic LAB include: safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonize the gastro-intestinal tract, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities, such as vitamin production, cholesterol assimilation and lactose activity [25]. The action of the antifungal properties of LAB on some mycotoxigenic fungi has been reported by a few authors, but the number of published studies on antifungal activity of LAB is still very low. A limited number of reports have shown that a good selection of LAB could allow the control of fungal growth and therefore reduce health risks due to exposure to mycotoxins [3,26]. There is an open area for research possibilities for prevention of fungal growth and elimination of mycotoxins from food or their transformation into less dangerous compounds, using the strains of lactic acid bacteria. The aim of this study was to evaluate the probiotic potential of *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casei*, *L. paracasei* and *Bifidobacterium bifidum* against several foodborne pathogenic fungi such as *Aspergillus niger*, *A. flavus*, *A. parasiticus* and *Penicillium chrysogenum* by examining their in vitro antimicrobial properties.

Materials and methods

Probiotic strains

L. acidophilus (LA-5), *L. rhamnosus* (LGG), *L. casei* (LC-01), *L. paracasei* and *B. bifidum* were purchased from CHR-Hensen Co., Denmark. Fully-grown bacterial colonies were stored on Mann Rogosa Sharpe (MRS) agar (BioMerieux, France) plates at 4 °C until used.

Fungal isolates

The food-spoiling fungi including *A. niger* (PTCC 5012), *A. flavus* (PTCC 5004), *A. parasiticus* (PTCC 5286) and *P. chrysogenum* (PTCC 5035) were obtained from Iranian Research Organization for Science and Technology (IROST).

Antifungal activity

Bacterial isolates were cultured on MRS after aerobic incubation for different *Lactobacillus* species and anaerobic incubation for *B. bifidum* at 37 °C for 48 h. The cell-free supernatant was prepared by MRS broth, centrifuging on 11,500×*g* for 10 min at 4 °C in order to remove the cells. Then, the supernatants were sterilized using 0.45 μm pore size filters (Biofil). Inhibitory activity of liquid cultures and supernatants obtained from 48 h-cultured probiotic bacteria were evaluated with agar well diffusion method, described by Guo et al. [12]. Briefly, fungal suspension, adjusted with 1×10^5 conidia/mL with Neubauer counting chamber, was mixed with MRS agar, dispended on plates and allowed to solidify. Then, wells with 5 mm diameters were made with Pasteur pipette in each plate. To cover the base of the wells, 20 μL of MRS agar were poured in each well. A volume of 100 μL of probiotic bacteria (106 colony forming unit (CFU)/mL) of a log-phase culture as well as the supernatant of probiotic bacteria were added to wells. The plates were incubated at 30 °C for 5 days. Inhibition of growth was determined by measuring the area of inhibition surrounding each agar well. All experiments were repeated on three separated occasions with triplicate determinations on each occasion. The antifungal activity of each probiotic bacteria against tested fungi were calculated according to the following formula:

$$FI(\%) = (IR/GR) \times 100;$$

FI: fungal inhibition; IR: inhibition radius; GR: growth radius.

Statistical analysis

All data were statistically analyzed with SPSS version 20, using one-way ANOVA (Tukey post-hoc) with 95% confidence level.

Results and discussion

The results of liquid cultures obtained from all bacterial isolates exhibited varying degrees of inhibitory activity against selected fungi (Table 1). The isolate *L. casei* (FI: 24.83%) exhibited the superior antifungal activity with inhibition zones of range 11.2–16.7 mm, followed by the isolates *L. acidophilus* (FI: 23.63%) and *L. paracasei* (FI: 20.58%). In the literature, most of the active antifungal strains were related to the *L. casei* group [4]. The least activity was recorded for the isolate *B. bifidum* (FI: 7.8%; inhibition zone of range 1.5–6 mm), while the isolate *L. rhamnosus* (FI: 17.4%; inhibition zone of range 8.3–11.7 mm) was moderately active against tested fungi. In the present study, *Lactobacillus* species inhibited tested fungal strains more than *B. bifidum*, which is in accordance with the study of Demerdash and Mostafa [8]. The differences in the size of inhibition zone were related to the strain's production of antimicrobial agents in addition to the production of acids, which decreased the pH value. Muhialdin et al. [20] and Magnusson et al. [18] demonstrated that activity of LAB was stable at pH 3–4.5 with maximum antifungal effect.

In this study, out of the five isolates, *L. casei* isolate showed maximum fungal inhibition against *P. chrysogenum* (28.9 ± 2.8%), followed by *A. niger* (26.9 ± 3.4%), *A. parasiticus* (24.1 ± 4.6%) and *A. flavus* (19.4 ± 1.3%); *L. acidophilus* isolate showed maximum fungal inhibition against *A. flavus* (28.4 ± 2.8%), followed by *A. parasiticus* (22.9 ± 2.8%), *P. chrysogenum* (22.6 ± 4.9%) and *A. niger* (20.6 ± 2.6%); *L. paracasei* isolate showed maximum fungal

Table 1 Antifungal activity of liquid cultures of probiotic bacteria against foodborne fungi.
Activité antifongique du liquide de culture des bactéries probiotiques contre les champignons de l'alimentation.

Probiotic	Fungi							
	Aspergillus niger		*Aspergillus flavus*		*Aspergillus parasiticus*		*Penicillium chrysogenum*	
	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)
L. rhamnosus	10.5 ± 0.8	18.3 ± 1.5 ^{aA}	11.7 ± 1.6	20.3 ± 2.8 ^{aAC}	8.3 ± 1.2	14.5 ± 2.1 ^{bB}	9.5 ± 1.1	16.5 ± 1.8 ^{abAC}
L. acidophilus	11.8 ± 1.5	20.6 ± 2.6 ^{aA}	16.3 ± 1.6	28.4 ± 2.8 ^{bB}	13.2 ± 1.6	22.9 ± 2.8 ^{aA}	13 ± 2.8	22.6 ± 4.9 ^{aAB}
L. casei	15.5 ± 1.9	26.9 ± 3.4 ^{aB}	11.2 ± 0.7	19.4 ± 1.3 ^{bC}	13.8 ± 2.6	24.1 ± 4.6 ^{abA}	16.7 ± 1.6	28.9 ± 2.8 ^{aB}
L. paracasei	11.7 ± 2.3	20.3 ± 3.9 ^{abcA}	14 ± 1.1	24.3 ± 1.9 ^{bAB}	11.5 ± 1.4	20 ± 2.4 ^{abcA}	10.2 ± 2.3	17.7 ± 4.0 ^{cA}
B. bifidum	5.7 ± 1.9	9.8 ± 3.4 ^{aC}	4.8 ± 1.7	8.4 ± 3 ^{abD}	1.5 ± 1.5	2.6 ± 2.6 ^{bC}	6 ± 3.2	10.4 ± 5.6 ^{aC}

Values with the same small letter in the same row were not significantly different ($P > 0.05$); values with the same capital letter in the same column were not significantly different ($P > 0.05$).

Table 2 Antifungal activity of the supernatants of probiotic bacteria against foodborne fungi.
Activité antifongique du surnageant de bactéries probiotiques contre les champignons de l'alimentation.

Probiotic	Fungi							
	Aspergillus niger		*Aspergillus flavus*		*Aspergillus parasiticus*		*Penicillium chrysogenum*	
	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)	ID (mm ± SD)	FI (% ± SD)
L. rhamnosus	7.7 ± 1.4	13.3 ± 2.4 ^{abA}	8.8 ± 0.9	15.4 ± 1.7 ^{bA}	7.0 ± 1.1	12.2 ± 1.9 ^{aB}	7.5 ± 1.1	13.1 ± 1.8 ^{abA}
L. acidophilus	9.8 ± 1.2	17.1 ± 2.0 ^{aB}	14.5 ± 2.6	25.2 ± 4.5 ^{bB}	12.3 ± 2.5	21.4 ± 4.3 ^{abA}	9.8 ± 0.7	17.1 ± 1.3 ^{aB}
L. casei	13.5 ± 1.5	23.5 ± 2.6 ^{acC}	10.0 ± 2	17.4 ± 3.5 ^{bA}	11.3 ± 1.5	19.7 ± 2.6 ^{abA}	14.3 ± 1.2	24.9 ± 2.1 ^{cC}
L. paracasei	9.5 ± 0.55	16.5 ± 0.1 ^{abAB}	11.7 ± 2.2	19.4 ± 3.9 ^{bAB}	9.8 ± 1.5	17.1 ± 2.6 ^{bA}	7.5 ± 0.8	13.4 ± 1.5 ^{aA}
B. bifidum	3.3 ± 1.3	5.8 ± 2.4 ^{abcD}	4.2 ± 2.9	7.2 ± 5.0 ^{bC}	0.8 ± 1.3	1.4 ± 2.3 ^{cC}	4.5 ± 2.1	7.8 ± 3.6 ^{abD}

Values with the same small letter in the same row were not significantly different ($P > 0.05$); values with the same capital letter in the same column were not significantly different ($P > 0.05$).

activity against *A. flavus* (24.3 ± 1.9%), followed by *A. niger* (20.3 ± 3.9%), *A. parasiticus* (20 ± 2.4%) and *P. chrysogenum* (17.7 ± 4.0%); *L. rhamnosus* isolate showed maximum fungal activity against *A. flavus* (20.3 ± 2.8%), followed by *A. niger* (18.3 ± 1.5%), *P. chrysogenum* (16.5 ± 1.8%) and *A. parasiticus* (14.5 ± 2.1%); and *B. bifidum* isolate showed maximum fungal activity against *P. chrysogenum* (10.4 ± 5.6%), followed by *A. niger* (9.8 ± 3.4%), *A. flavus* (8.4 ± 3.0%) and *A. parasiticus* (2.6 ± 2.6%). Altogether, *A. flavus* (FI: 20.16%) was the most susceptible fungal strain to probiotic bacteria, followed by *P. chrysogenum* (FI: 19.22%), *A. niger* (FI: 19.18%) and *A. parasiticus* (FI: 16.82%). The agar well diffusion method, used in this test, proved to be useful for selecting probiotic isolates of *Lactobacillus* and *Bifidobacterium* species that possess the ability to inhibit the fungal pathogens. There have been several reports on antifungal properties of lactobacilli; e.g. *L. acidophilus* [5,15,23], *L. casei* [11,29], *L. rhamnosus* [5,27,29], *L. paracasei* [6,13] and *B. bifidum* [1,6]. Magnusson et al. [18] tested the antifungal activity of a large number of *Lactobacillus* isolates and demonstrated strong inhibitory activity against different *Aspergillus* and *Penicillium* species. Similarly, Elbadry [7] tested the antifungal activity of five lactobacilli against four pathogenic fungi (*Rhizoctonia*, *Sclerotium*, *Fuzarium* and *Penicillium*). He found that the crude cell-free culture supernatants showed variations in their antifungal activity of range 48–63% fungal inhibition zone, and *Penicillium* species was the most susceptible indicator fungi [7]. In addition, El-Nezami et al. [9] demonstrated that LAB are efficient to inhibit the mycotoxigenic fungi such as *P. expansum*, *Botrytis cinerea*, *A. niger*, *A. flavus* and *Fusarium graminearum*. Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms [31]. Organic acids, such as lactic, acetic, propionic and phenyllactic acids, have frequently been involved in the antifungal activity of LAB [17]. In a study by Lavermicocca et al. [16], phenyllactic acid was able to inhibit the growth of *P. expansum*, *A. niger*, *A. flavus* and *F. graminearum* at a concentration of about 50 mg/mL.

As illustrated in Table 2, the antifungal activity of the supernatants obtained from all probiotic isolates against fungi was significantly less than that of liquid cultures ($P < 0.05$). The isolate *L. casei* (FI: 21.38%) possessed the highest activity, while the isolate *B. bifidum* (FI: 5.6%) was highly significantly the least active ($P < 0.05$). *A. flavus* (FI: 16.92%) was the most susceptible fungal strain to bacterial supernatants, followed by *P. chrysogenum* (FI: 15.26%), *A. niger* (FI: 15.24%) and *A. parasiticus* (FI: 14.36%). Our results agree with those reported by Vanne et al. [32], who assayed the effect of *L. casei* on growth of *A. flavus*. The displayed strong ability to inhibit species of *A. flavus* is a very promising result and a good testimonial since this fungus often shows resistance [13] and the inhibition of its growth is usually a difficult task. In addition, *A. flavus* is now considered as the leading cause of aspergillosis in human and among the most toxic and carcinogenic representatives of the micromycetes [14,22]. It is worth mentioning that the inhibitory activity of the tested isolate supernatants was less against fungi when compared to that obtained by liquid cultures, indicating that supernatants could be less active. A similar result was observed by Donkova et al. [6] who exhibited greater antifungal activity of liquid cultures than that of the supernatants of some *Lactobacillus* species. It means that the *Lactobacillus* strains' inhibition is not only due to the formation of lactic acid and other organic acids resulting in decreased pH, but there is also a competition for nutrients between the *Lactobacillus* strain and the fungi as well as production of metabolites with antimicrobial effect by the *Lactobacillus* strain.

Conclusions

In summary, this study exhibited that the selected lactic acid bacteria, in particular *L. casei*, *L. acidophilus* and *L. paracasei*, had excellent probiotic characteristics and thus can be used as potential sources of probiotic. Study affirms their use in the development of new pharmaceutical preparations and functional foods belonging to vegetables and fruits as probiotics for the betterment of public health. Further investigations to elucidate the nature of inhibiting compounds should be considered.

Disclosure of interest

The authors declare that they have no competing interest.

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