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Antifungal Activity of Selected Lactobacilli Intended for Sourdough Production

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Abstract

Background and objective: Presently, there is a growing interest in food produced without the addition of chemical preservatives. Lactic acid bacteria have a high potential to control the growth of undesirable microorganisms. In this work, the antifungal activity of eight strains of lactobacilli isolated from plant sources and foodstuffs (tartar sauce, wheat flour, barley flour, sourdough) were tested against *Fusarium culmorum* DMF301 and *Penicillium expansum* DMF04.

Materials and methods: Antifungal activity of live cells of lactobacilli and their heat-treated supernatants was determined by agar diffusion method at 30°C, the radial growth of fungi was measured. Organic acid production of tested strains was examined by HPLC.

Results and conclusion: Live cells of three *Lactobacillus plantarum* strains showed the highest antifungal activities. *Fusarium culmorum* DMF301 was more sensitive to the activity of lactobacilli. Heat-treated bacterial supernatants (100°C, 10 min) were also tested, being added at 10 or 20% v v^{-1} to the culture medium; growth of *Fusarium culmorum* DMF301was inhibited by range of 80-90% compared to controls at 10% supernatant concentration and fully at 20%. However, after neutralization, only the heat treated supernatant from *Lactobacillus plantarum* CCDM 583 had partially effective antifungal activity. The mutual inhibition of lactobacilli strains reduced their antifungal activity. Statistically significant differences in activity against *Fusarium culmorum* DMF 301 were found using individual strains of *Lactobacillus plantarum* CCDM 583 and MP2 or their combination. For use in combination with other cultures, it is therefore necessary to verify the compatibility of various strains of lactobacilli.

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1. Introduction

Functional properties of lactic acid bacteria (LAB) have been traditionally applied not only in fermented dairy products but also in fermented vegetables, in sourdough and subsequently in bakery products. During fermentation, LAB produce a variety of metabolites (organic acids, bacteriocins, reuterin, hydrogen peroxide, cyclic peptides, ethanol, diacetyl etc.) showing both antibacterial and antifungal activity and these have been used successfully in the natural preservation of foods as a partial replacement of chemical preservatives [1,2].

The number of LAB isolates from sourdough includes more than 80 species of bacteria, with a significant number of strains from the families Lactobacillaceae and Leuconostocaceae, but lactococci, streptococci and enterococci are also present [3]. *Lactobacillus* (*L.) plantarum* and *L. sanfranciscensis* have been found in 50% of sourdoughs tested [4]. However, microbial metabolic activity, in combination with the enzymatic activity of the cereal substrates, has a significant effect on the quality of the resulting bakery product. The basic biochemical processes that take place during the preparation of sourdoughs are alcoholic and lactic fermentation, but different LAB present may also differ with other metabolic activities such as the ability to effectively utilize maltose or the formation of exopolysaccharides from sucrose [3,5,6].

Organic acids occur in foods as additives or as products of carbohydrates or protein metabolism. Lactic acid is the main product of carbohydrate metabolism by LAB; acetic

acid is produced in relatively large amounts only in the hetero-fermentative strains, and is a very important component involved in the taste and smell of sourdough [7,8]. The conversion of free fatty acids to antifungal-active hydroxy-fatty acids is a strain specific property of some LAB [9]. Phenylalanine and tyrosine may be degraded to phenyllactic acid and its p-hydroxy-derivative which inhibits mould growth at low concentrations [10].

The mechanism of antimicrobial action of organic acids is mainly acidification of the cytoplasm. The undissociated forms of acids diffuse through the microbial cell wall due to their hydrophobic character and then dissociate inside the cell. This phenomenon leads to inhibition of metabolic activities and the collapse of proton motive force. This mechanism is also likely to affect some types of fungi [11]. Using scanning and transmission electron microscopy has shown that *L. plantarum* K35 supernatant causes damage to the fungal cell wall and cytoplasmic membrane, as well as destruction of organelles including mitochondria and the nucleus [12]. Antifungal effects of cyclic peptides, hydrogen peroxide and the synergistic effects of various agents have also been investigated [1,2,10,13].

Selecting suitable protective cultures can have a number of positive effects since filamentous fungi are widespread food spoilage microorganisms responsible for economic losses and safety concerns due to the potential production of mycotoxins. This study focused on antifungal activities of selected lactobacilli against *Fusarium* (*F.*) *culmorum* DMF301 and *Penicillium* (*P.*) *expansum* DMF04 isolated from bakery products. *Fusarium* spp. and *Penicillium* spp. were identified as frequent fungal contaminants in food, causing spoilage and synthesizing highly toxic mycotoxins [14]. The aim of this work was also to verify the stability of the antifungal activity at baking temperatures. Additionally, the antifungal activity of the individual strains and their combinations was compared.

2. Materials and methods

Microorganisms

Lactobacilli were obtained from Laktoflora®Milcom collection of microorganisms (Prague, Czech Republic). *L. acidophilus* CCDM 151 (original culture); *L. plantarum* CCDM 181 (source silage); *L. zymae* CCDM 361 (isolated from wheat); *L. sanfranciscensis* CCDM 451; *L. panis* CCDM 471; *L. sanfranciscensis* CCDM 827 (all isolated from sourdough); *L. plantarum* CCDM 583 (isolated from barley flour); and *F. culmorum* DMF301; *P. expansum* DMF04 and *L. plantarum* MP2 (isolated from tartar sauce) were obtained from the collection of the Department of Dairy, Fat and Cosmetics, University of Chemistry and Technology (Prague, Czech Rep.).

Cultivation of microorganisms

Lactobacilli strains were routinely cultivated in MRS broth (Merck, Germany) at 37°C for 18 h in an atmosphere of 5% v v^{-1} CO_2. After cultivation, the numbers of lactobacilli were determined by plating on MRS agar (Merck, Germany), pH 5.8 at 37°C in an atmosphere of 5% v v^{-1} CO_2.

Both fungi were plated on yeast extract sucrose slant agar (YESA) containing yeast extract 20 g (Oxoid, Altrincham, UK), sucrose 150 g (VWR, Oud-Heverlee, Belgium) magnesium sulphate heptahydrate 0.5 g (Lachema, Czech Rep.), zinc sulphate heptahydrate 0.01 g (Penta, Prague, Czech Rep.), copper sulphate pentahydrate 0.005 g (Lachema, Czech Rep.), agar 20 g (Oxoid, Altrincham, UK) and 885 ml water, pH 5.7, and incubated at room temperature aerobically for 10 days.

Preparation of spore suspension

A test-tube with slant agar was washed with 5 ml of saline solution containing 0.1% w v^{-1} peptone and 0.1% v v^{-1} Tween 80. The spore concentration in this solution was determined by cultivation method and subsequently adjusted by dilution with saline solution to approximately 10^5 per ml and stored at 8°C until further use.

HPLC analysis of organic acids

Determination of organic acids was performed by HPLC (Agilent 1260 Infinity, USA): Polymer IEX H column (Watrex, Prague, Czech Rep.) 250 × 8 mm and pre-column 40 × 8 mm (Watrex) both with particle size 8 µm, temperature 60°C, mobile phase sulfuric acid (9 mmol l^{-1}), flow rate 0.6 ml min^{-1}; sample volume 20 µl; detection was by UV detector at 210 nm.

Antifungal activity experiment

Antifungal activity was determined using an agar diffusion method [15]. 100 µl of appropriate dilution of lactobacilli suspension were mixed with 10 ml of modified (acetate-free) MRS agar to a final concentration of 10^2-5×10^2 CFU ml^{-1} and poured on Petri dishes (with a diameter of 8.5 cm). After solidification, the plates were overlaid with 5 ml of YESA soft agar (0.75% agar), dried and 5 µl of spore suspension of *F. culmorum* DMF301 or *P. expansum* DMF04 were spotted in the middle of the plate. Radial growth of moulds was measured regularly in two different directions during aerobic cultivation at 30°C. In order to evaluate the activity of cell free supernatants, the lactobacilli cells after cultivation were centrifuged (9000 ×g, 4°C, 5 min), supernatants were heat-treated (100°C, 10 min) and then either neutralized to pH 7 with 10% w w^{-1} NaOH, or used without neutralization. Both types of supernatants (heat-treated and heat-treated with neutralization) were mixed with YESA to achieve a final concentration of 10 or 20% v v^{-1} in agar. Subsequent fungal cultivation was carried out for 10 days at 25°C and radial growth of the mould was monitored by measuring the diameter of the colony using a digital sliding scale. As

a control, growth of mould on agar without the addition of lactobacilli or supernatants was used.

Statistical analysis

All measurements were means from three independent trials with three parallel repetitions (n=9). Standard statistic methods in Excel (Microsoft® Office 2010) were used to evaluate the results: F-test to assess the equality of variances and student´s paired t-test to evaluate the means significant differences. Differences were considered significant at $p \leq 0.05$.

3. Results and discussion

Tested strains of lactobacilli were characterized by growth in MRS broth, acid production and final pH after 18 h cultivation. The results are shown in Table 1. All strains showed final cell concentrations of 10^8-10^9 CFU ml^{-1} except strains 451 and 471 (only 10^5-10^6 CFU ml^{-1}). These strains differed in acid production. Strains 361 and 583 produced the largest amount of lactic and acetic acids. In case of the strain 361, it was 38.6±1.7 and 4.0±0.2 g l^{-1} and in the case of the strain 583 it was 38.0±2.4 and 3.7±0.1 g l^{-1} respectively. Although strain 151 belongs to the homo-fermentative group of lactobacilli, it was found to produce little acetic acid. Production of organic acids (lactic, acetic, phenyllactic) is the main reason for inhibition of mould growth [16]. Trace amounts of phenyllactic acid were only found in MP2 and 361. Its production could however be increased by the addition of phenylalanine to the cultivating medium as a precursor [17]. Surprisingly, low levels of acetic acid were found in the obligatory hetero-fermentative strain CCDM 827. The formation of antifungal compounds depends on the growth and metabolic activity of LAB. Basic factors influencing LAB growth and the level of antifungal metabolites include temperature, incubation time, nutritional factors and the pH of the culture medium [12,14,18].

The radial growth of fungi in the presence of a growing culture of lactobacilli during 10 days cultivation at 30°C is summarized in Fig. 1 and Fig. 2, being compared to control growth (without lactobacilli). *Fusarium* spp. and *Penicillium* spp. and their mycotoxins are of the most common contaminants of cereals [2]. They pose not only a health risk for humans and animals but also influence technological and baking properties of dough [19].

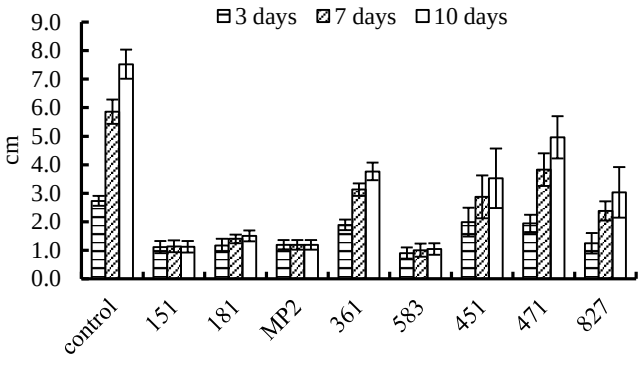

Figure 1. The radial growth of *Fusarium culmorum* DMF01 in the presence of growing cells of lactobacilli at 30°C

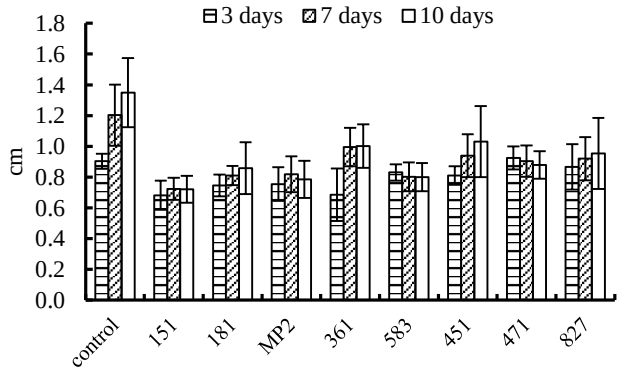

Figure 2. The radial growth of *Penicillium expansum* DMF01 in the presence of growing cells of lactobacilli at 30°C

Table 1. Growth characteristics and acid production of tested lactobacilli after cultivation in MRS broth at 37°C, 18 h, 5% v v^{-1} CO_2

Strain	Cell count [CFU ml^{-1}]	pH	Lactic acid [g l^{-1}]	Acetic acid [g l^{-1}]	Phenyllactic acid
Lactobacillus acidophilus CCDM 151	$4.6±0.2·10^9$	4.52	28.5±0.3	0.3±0.0	ND
Lactobacillus plantarum CCDM 181	$2.9±0.1·10^9$	4.01	36.3±2.1	2.8±0.1	ND
Lactobacillus plantarum MP2	$3.1±0.3·10^9$	3.78	34.1±2.8	2.7±0.1	Traces
Lactobacillus zymae CCDM 361	$8.1±0.3·10^8$	3.75	38.6±1.7	4.0±0.2	Traces
Lactobacillus sanfranciscensis CCDM 451	$3.8±0.5·10^5$	4.25	13.5±0.7	2.9±0.1	ND
Lactobacillus panis CCDM 471	$2.3±0.4·10^6$	5.04	29.1±1.1	0.9±0.0	ND
Lactobacillus plantarum CCDM 583	$6.5±0.2·10^8$	3.81	38.0±2.4	3.7±0.1	ND
Lactobacillus sanfranciscensis CCDM 827	$8.6±0.1·10^8$	4.94	29.8±1.7	0.8±0.0	ND

Growing cells of lactobacilli were able to inhibit the growth *F. culmorum* DMF301 much more than *P. expansum* DMF04. The results shown in Fig. 1 and Fig. 2 demonstrate that *L. plantarum* strains CCDM 181, CCDM 583, MP2 and *L. acidophilus* CCDM 151 had the greatest inhibitory effects (about 80-86% inhibition when compared to the control growth after 10 days) on growth of *F. culmorum* DMF301, whereas the inhibitory activity against *P. expansum* DMF04 was lower and reached only 36-46%. On the other hand, the weakest antifungal activity against moulds was observed using strains 361, 451 and 471 (about 35-53% inhibition activity against *F. culmorum* DMF301 and 23-34% against *P. expansum* DMF04). The antifungal effect of lactobacilli is described in a number of studies. Stiles et al. [15] verified the antifungal effect of *L. rhamnosus* VT1 against *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp. They found that sodium acetate, which is one of the components of MRS agar, exhibits antifungal activity. They noted that this strain showed a synergistic effect with sodium acetate. Also, Schillinger and Villarreal [20] proved the antifungal effect of MRS medium on *P. nordicum* using different concentrations of sodium acetate. For this reason, modified MRS agar without sodium acetate was used in this work. Yang and Chang [21] recorded the ability of *L. plantarum* AF1 to inhibit the growth of *Aspergillus* sp., *Cladosporium* sp., *Epicoccum* sp. and *Penicillium* sp. Demirba et al. [22] confirmed the antifungal effect of *L. paraplantarum* N-15 and *L. paralimentarius* E-106 on *A. niger* and *P. chrysogenum*.

Bread could also be an important non-dairy vehicle for LAB since some of the bacteria can survive the high baking temperatures, as was proven in several studies. After baking, Zhang et al. [23] found 10^{4-5} CFU g^{-1} live cells of *L. plantarum* P8, the number of which increased up to 10^8 CFU g^{-1} in the crust and 10^6 CFU g^{-1} in the crumb during storage. Nevertheless, during the baking process, live lactobacilli are mostly inactivated. Antifungal activity is then caused by stable antifungal metabolites. The temperature inside the crumb usually does not exceed 90-100°C and therefore heating at 100°C was chosen to produce heat-treated supernatants [24]. The plates of YESA were supplemented with 10 or 20% v v^{-1} heat-treated cell free supernatant (CFS) of strains with good growth in MRS broth (151, 181, MP2, 361, 583, 827) to test their activity against *F. culmorum* DMF301. A 10% addition of CFS meant an approximate acid concentration (lactic and acetic) produced by LAB of about 0.37-0.43 g to 100 g of agar and a 20% addition about 0.74-0.85 g to 100 g of agar. Strong inhibitory activity (80-90% inhibition) was observed when the medium was supplemented with either 10% or 20% v v^{-1} heat-treated CFS of strains 361, 583 and 827. A concentration of 20% v v^{-1} was required to observe a reduction in growth of the mould (approximately 90%) for strains 151, 181 and MP2. In the case of 10% supplementation with CFS, only 18%, 46% and 58% reduction respectively was detected after 7 days cultivation. The conclusion that the cell-free heat treated supernatants of four LAB (80°C for 1 h or 121°C for 15 min) were inhibitory against *P. notatum* was also published by Rouse et al. [25]. Similar results were confirmed for heat treated supernatants (exposure to a temperature of 100°C for 10 min) of different strains of Lactobacillus against *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp [26]. Heating, refrigeration or freezing did not change the supernatant inhibitory activity of lactobacilli against different moulds [27]. In the present study, no inhibition was detected when neutralized heat-treated cell free supernatants were added to the agar at a final concentration of either 10% v v^{-1} or 20% v v^{-1} except for strain 583. As is shown in Fig. 3 and Fig. 4, neutralized supernatant (20%) of strain 583 was able to significantly inhibit the growth of *F. culmorum* DMF301 but not *P. expansum* DMF04 at 25°C.

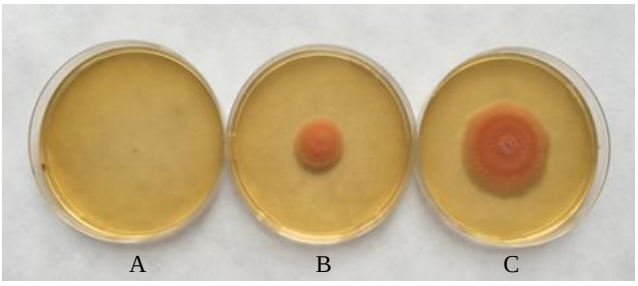

Figure 3. Growth of *Fusarium culmorum* DMF301 on YESA supplemented with 20% v v^{-1} heat-treated CFS (A) and neutralized heat-treated CFS (B) of *Lactobacillus plantarum* CCDM 583 and the control growth without cell free supernatant (C) of the mould after 5 days at 25°C.

The ability of heat-treated cell-free supernatants to inhibit fungal growth has been described in a number of studies. Organic acids are responsible for the antimicrobial effects of non-neutralized cell-free supernatants. The same conclusion was drawn by Gerez et al. [28] who found that the cell-free supernatants of strains of lactobacilli had lost their antifungal activities after neutralization. De Muynck et al. [29] noted that strains belonging to the species *P. paneum* and *P. roqueforti* were very susceptible to the action of the cell-free supernatant of the *L. acidophilus* LMG9433. They further demonstrated that after neutralization cell-free supernatants of *L. acidophilus* LMG9433, *L. amylovorus* DSM20532, *L. brevis* LMG6906 and *L. coryniformis* subsp. *coryniformis* LMG9196 lost their antifungal effects. Le Lay et al. [30] noted that the cell-free supernatants of *L. br*evis Lu35 and *L. citreum* L123 showed strong growth inhibition of *A. niger* UBOCC-A-112064 and *P. corylophilum* UBOCC-A-112081.

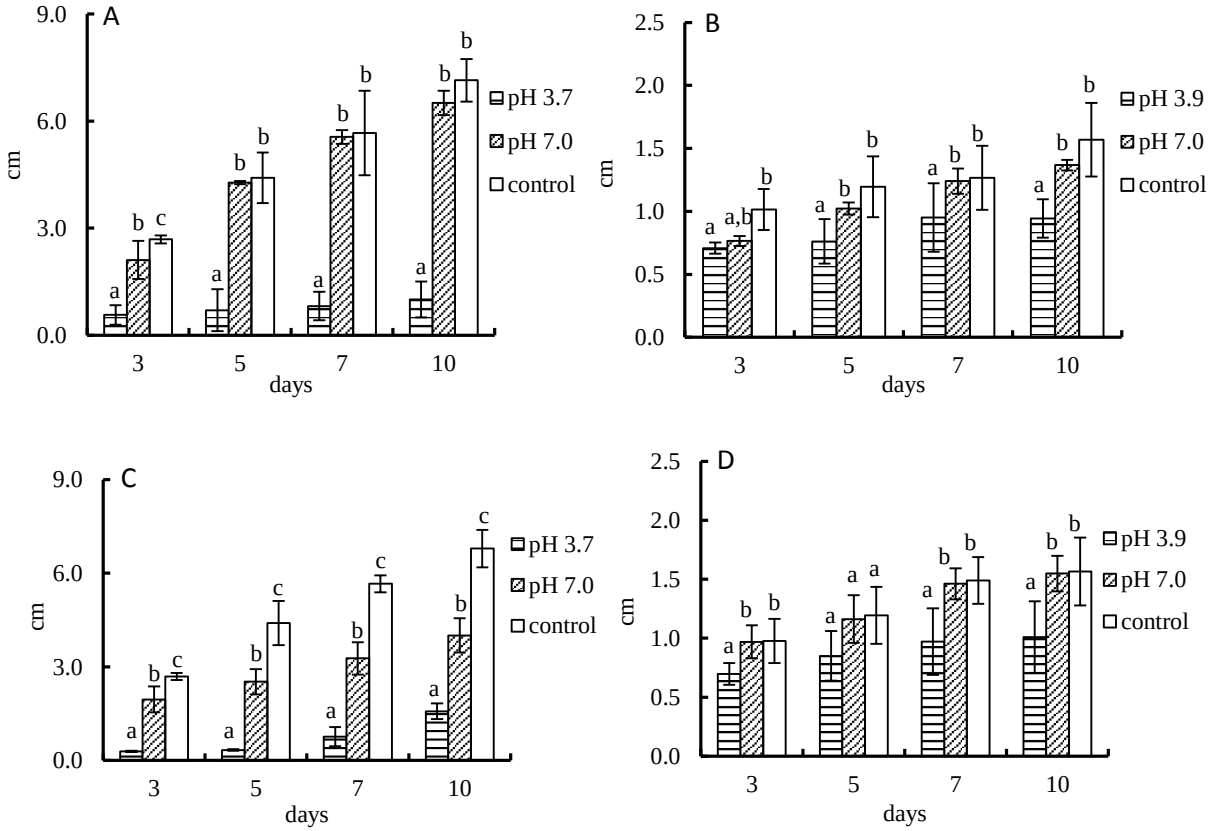

Figure 4. The radial growth of mould *Fusarium culmorum* DMF301 (A,C) and *Penicillium expansum* DMF04 (B,D) on YESA supplemented with 10% v v^{-1} heat-treated CFS (pH 3.7 or 3.9) or neutralized heat-treated CFS (pH 7) of *Lactobacillus zymae* CCDM 361 (A,B) and *Lactobacillus plantarum* CCDM 583 (C,D) at 25°C.

Different letters in the columns of particular days indicate significant differences ($p \leq 0.05$) among samples.

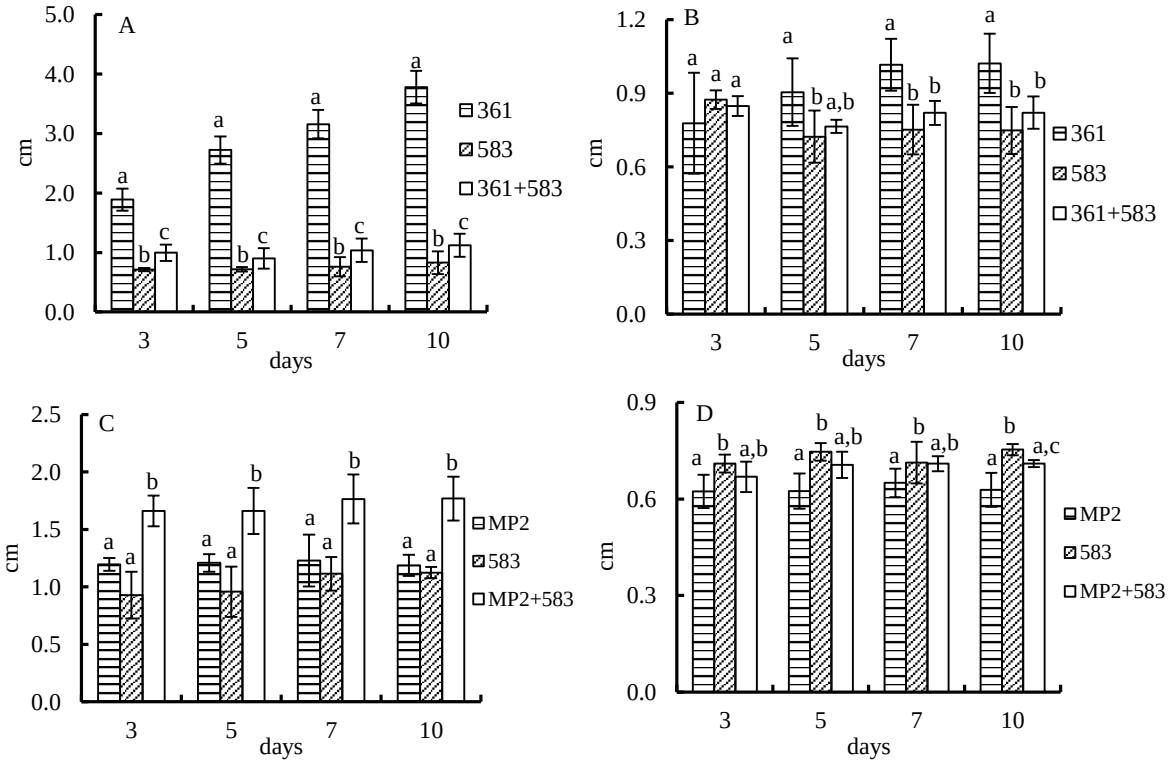

Figure 5. The radial growth of *Fusarium culmorum* DMF301 (A,C) and *Penicillium expansum* DMF04 (B,D) in the presence of single strains *Lactobacillus zymae* CCDM 361, *Lactobacillus plantarum* MP2 and *Lactobacillus plantarum* CCDM 583 and their combination at 30°C.

Different letters in the columns of particular days indicate significant differences ($p \leq 0.05$) among samples.

Since protective adjunct cultures can often be used in mixtures, the antifungal effect of selected combinations of lactobacilli strains, i.e. combination of 361 and 583 and combination of MP2 and 583, were investigated in further work. The final concentration of live cells was again 10^2-5×10^2 CFU ml^{-1} with the use of a 50% inoculum of each strain. Synergistic effects of the strains were expected but, as shown in Fig. 5, the efficiency of *L. plantarum* CCDM 583 against *F. culmorum* DMF301 was reduced when grown together with strains 361 and MP2. This phenomenon was not observed in tests of antifungal activity against *P. expansum* DMF04. Mutual inhibition was confirmed in all three strains of lactobacilli tested against each other. The zones of inhibition, determined by agar diffusion method, are shown in Table 2 and Fig. 6. The results show that careful selection of mutually compatible strains is necessary to ensure their desired properties. It is important especially in the cases where other dairy cultures are used for food production. The antifungal activity of lactobacilli can successfully be utilized not only to suppress mould growth in sourdough breads, but in a variety of other applications as well [31]. Antifungal LAB were tested for prevention of mould on fresh vegetables or apples and *L. plantarum* strains were able to inhibit spoilage yeast in refrigerated foods or to inhibit fumonisin producing *F. proliferatum* in poultry feeds [18,25,32,33]. Antifungal cultures have also been introduced for yoghurt production and can be a good choice to increase the shelf life of cereal-based beverages [34,35].

Table 2. Inhibition zones (mm) found by agar diffusion method after common cultivation of lactobacilli strains *Lactobacillus zymae* CCDM 361, *Lactobacillus plantarum* MP2 and *Lactobacillus plantarum* CCDM 583 at 37°C, 48 h, 5% v v^{-1} CO_2 atmosphere

Inhibited by strain	361	583	MP2
361	-	15.33±0.31	15.55±0.18
583	14.33±0.33	-	18.15±0.38
MP2	15.30±0.18	18.10±0.21	-

361= *Lactobacillus zymae* CCDM 361, 583= *Lactobacillus plantarum* CCDM 583, MP2= *Lactobacillus plantarum* MP2

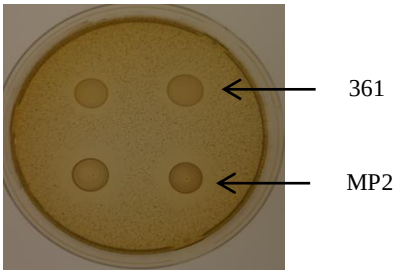

Figure 6. Inhibition zones of the strain *Lactobacillus zymae* CCDM 361 and *Lactobacillus plantarum* MP2 against the strain *Lactobacillus plantarum* CCDM 583; cultivation at 37°C, 48 h, 5% v v^{-1} CO_2

4. Conclusion

The use of antimicrobial properties of LAB is an interesting alternative and could replace artificial chemical preservatives, as is frequently demanded by many consumers. Based on the results obtained in this work, *L. plantarum* strains CCDM 181, CCDM 583 and MP2 can be recommended as potential antifungal agents for further application because their activity against selected mould was confirmed. When used, their compatibility with regard to antifungal activity must be taken into account. Different lactobacilli strains may inhibit each other in mixed cultures, which may lead to a subsequent reduction of their antifungal effect.

5. Acknowledgement

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6. Conflict of interest

The authors declare no conflict of interest.

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فعالیت ضدقارچی لاکتوباسیل‌های انتخابی برای تولید خمیر ترش

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چکیده

سابقه و هدف: اخیراً علاقه روزافزونی در زمینه تولید مواد غذایی بدون نگهدارنده‌های شیمیایی ایجاد شده است. باکتری‌های اسید لاکتیک توانایی زیادی در کنترل رشد ریزاندامگان ناخواسته دارند. در این کار، فعالیت ضدقارچی هشت سویه لاکتوباسیل جدا شده از منابع گیاهی و مواد غذایی (سس تارتار، آرد گندم، آرد جو، خمیر ترش) در برابر *فوزاریوم کالموروم DMF301* و *پنی سیلیوم اکسپانسوم* بررسی شد.

مواد و روش‌ها: فعالیت ضدقارچی سلول‌های زنده لاکتوباسیل و مایع رویی حرارت دیده آنها با روش انتشار در آگار در درجه حرارت ۳۰ درجه سلسیوس تعیین و هاله رشد اندازه‌گیری شد. تولید اسید آلی سویه‌های مورد آزمون با HPLC بررسی شد.

یافته‌ها و نتیجه‌گیری: سلول‌های زنده سه سویه *لاکتوباسیلوس پلانتاروم* بیشترین فعالیت ضدقارچی را از خود نشان دادند. *فوزاریوم کلاموروم* DMF301 به فعالیت لاکتوباسیل‌ها بیشتر حساس بود. مایع رویی باکتری‌های حرارت دیده (۱۰۰°C، ۱۰ دقیقه) نیز مورد آزمون قرار گرفت و به میزان ۱۰ یا ۲۰ درصد حجمی-حجمی به محیط کشت اضافه شد؛ رشد *فوزاریوم کلاموروم* DMF301 به میزان ۸۰ تا ۹۰ درصد در غلظت ۱۰ درصد مایع رویی و به طور کامل در غلظت ۲۰ درصد آن مهار شد. هرچند، پس از خنثی‌سازی فقط مایع رویی حرارت دیده *لاکتوباسیلوس پلانتاروم* CCDM 583 فعالیت ضدقارچی نسبتاً مؤثری داشت. اثر مهارکنندگی مشترک سویه‌های لاکتوباسیل فعالیت ضدقارچی آنها را کاهش داد. در فعالیت سویه‌های *لاکتوباسیلوس پلانتاروم* CCDM 583 و MP2 به تنهایی یا در ترکیب با هم در برابر *فوزاریوم کلاموروم* DMF301 تفاوت آماری معنی دار یافت شد. برای استفاده در ترکیب با سایر کشت‌ها لازم است سازگاری سویه‌های گوناگون لاکتوباسیل مورد تایید قرار گیرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- استیک اسید
- *فوزاریوم کلاموروم*
- اسید لاکتیک
- لاکتو باسیلوس
- *پنی‌سیلیوم اکسپانسوم*

***نویسنده مسئول**

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