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Potential of lactic acid bacteria in biocontrol of *Aspergillus niger, Penicillium chrysogenum* and *Fusarium graminearum* in culture media and natural substrate

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Abstract

Adverse storage conditions impair the quality of stored grain, contribute to the growth and propagation of the fungi leading to an even greater deterioration of the grain quality. Antifungal biopreparations can help preserve grain quality. Lactic acid bacteria (LAB) and their metabolites with antifungal properties can potentially be used in storage conditions or for the decontamination of stored products. The aim of this study was to determine the antifungal activity of *Lactobacillus casei*, *Lactobacillus brevis* and *Leuconostoc mesenteroides* on the inhibition of mycelial growth of pathogenic fungi *Aspergillus niger*, *Penicillium chrysogenum* and *Fusarium graminearum.* The biocontrol potential of LAB against fungi was tested under laboratory conditions in culture media as an ideal substrate for fungal growth and wheat grains as a model of natural substrate. A liquid culture media (MRS broth) was inoculated with cells or cell-free supernatants (CFS) of each LAB species and the fungal spores. The fungal growth was evaluated by measuring the increase in mycelial biomass after 7, 14, 21 and 28 days of incubation. The second antifungal assay was performed on the wheat grain treated with LAB CFS and inoculated with fungal spores. Biopreparation containing cells or CFS of all LAB species significantly inhibited the fungal growth of *P. chrysogenum* (mean inhibition 69–75% LAB cells and 80–81% LAB CFS) and *F. graminearum* (mean inhibition 60–83% LAB cells and 83–88% LAB CFS) in the culture media. In the treatment with wheat grain, the CFS of all the tested LAB species significantly inhibited only the growth of *F. graminearum* (83–90% mean inhibition). There is inconsistency in the efficiency of LAB preparations when comparing assays. The results indicate that media used in the experiment affect the activity or tolerance of the tested bacteria and fungi. *L. casei*, *L. brevis* and *L. mesenteroides* proved their antifungal properties in the culture media and natural substrate. *F. graminearum* was the most susceptible, and *A. niger* was the most tolerant to treatments with LAB cells and LAB CFS.
More research is needed to reveal the mode of action of the LAB against phytopathogenic fungi in different conditions for their application on stored grains.

Key words: *Lactobacillus brevis*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, antifungal activity, wheat grain.

Introduction

The microbial population of stored grain is primarily dominated by fungi (Los et al., 2018). Fungal growth depends on the nutritional composition of grain and abiotic and biotic factors (Atanda et al., 2011). Further, biotic and microbial activity can cause undesirable effects on grain including discoloration, loss of dry matter and germination, which leads to reduced quality and represents a significant risk to the food chain (Magan, Aldred, 2007). Likewise, such conditions can contribute to growth and propagation of fungi. The most common contaminants of crops and stored grains are fungi belonging to orders *Aspergillus*, *Penicilium* and *Fusarium* (Atanda et al., 2011; Ćosić et al., 2012; Fleurat-Lessard, 2017; Mannaa, Kim, 2017). These fungi are also mycotoxin producers, whose ingestion causes acute or chronic toxicosis in humans and animals. Post-harvest treatment before and during storage is important in the prevention of such fungal spoilage and mycotoxin biosynthesis (Schmidt et al., 2018).

The main focus is on the prevention of fungal activity in the chain from farm to table; however, if contamination occurs during storage, decontamination or

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detoxification procedures can be used (Fleurat-Lessard, 2017). Decontamination includes physical, biological and chemical treatments. Consumer requirements are focused on foods with minimum or no chemical residues; therefore, physical and biological (microbiological) treatments are the main tools in post-harvest control of microbial spoilage in cereals (Liška et al., 2017; Schmidt et al., 2018). Microbiological treatments must satisfy certain criteria: in general, they must be efficient, harmless to humans and animals, and the principles of sustainable agriculture and consumer demands insist on environmental acceptability. Biopreparations meet these criteria. The use of biopreparations in combination with other protection measures would meet the requirements for the production of health-safe agricultural products and food in general (Martinez, 2015). Biopreparations in the control of phytopathogenic fungi can be based upon antagonistic microorganisms or their metabolites (Gomah, Zohri, 2014).

Lactic acid bacteria (LAB) and their metabolites could act as bioagents and replace traditionally used chemical products in pest management (Schnürer, Magnusson, 2005). In fermented food, LAB produce organic acids, ethanol, hydrogen peroxide and bacteriocin with antimicrobial properties (De Vuyst, Leroy, 2007). The antifungal efficacy of LAB was confirmed in a series of studies (Magnusson et al., 2003; Muñoz et al., 2010; Schillinger, Villarreal, 2010; Matei et al., 2014).

An antagonistic LAB strain against *Aspergillus ochraceus* was identified in an assay on agar medium and recommended to be used as a biocontrol agent (Matei et al., 2014). *Lactobacillus fermentum* and *L. rhamnosus* showed fungal growth inhibition of *Aspergillus nomius* on plates using an overlay technique (Muñoz et al., 2010). Antifungal properties of LAB against *Penicillium nordicum* on agar plates were also determined (Schillinger, Villarreal, 2010). Some of LAB isolates are able to inhibit mycotoxin synthesis *in vitro* and *in vivo* (Oluwafemi et al., 2010; Franco et al., 2011; Zou et al., 2012). LAB produce a diversity of antimicrobial compounds affecting the populations of bacterial and fungal phytopathogens of crop plants; therefore, it is recommended as a probiotic in different agricultural ecosystems (Oliveira et al., 2014; Fleurat-Lessard, 2017; Lamont et al., 2017). The use of LAB and their metabolites as a potential measure in prevention of grain deterioration or decontamination in storage conditions should also be considered.

Considering the growing interest in the use of natural and with low ecological impact antimicrobial compounds in agricultural production, storage and decontamination, the experiment to test the antifungal activity of LAB was conducted. The objectives of the study were to determine the effect of *Lactobacillus brevis*, *L. casei* and *Leuconostoc mesenteroides* and their metabolic products in cell-free supernatants on the growth of frequent storage fungal contaminants *Penicillium chrysogenum*, *Aspergillus niger* and *Fusarium graminearum* in culture media and wheat grain as a natural substrate.

Materials and methods

Microbial isolates and growth media. Lactic acid bacteria (LAB) used in the experiment were: *Lactobacillus casei*, *Lactobacillus brevis* and *Leuconostoc mesenteroides*, previously isolated from diary and fermented products at the Faculty of Agrobiotehnical Sciences, J. J. Strossmayer University of Osijek, Croatia. LAB isolates were cultivated on MRS (De Man, Rogosa and Sharpe) broth 48 h at 37°C temperature. For experimental purposes, LAB cell-free supernatants (CFS) were prepared by centrifuge Centric 150 (Domel d.o.o., Slovenia) at 10,000 rpm for 15 min and sterilized by filter (Sartorius GmbH, Germany) through 0.2 μm pore size.

Fungal isolates of *Penicillium chrysogenum* and *Aspergillus niger* previously isolated at the Faculty of Agrobiotechnical Sciences, J. J. Strossmayer University of Osijek, Croatia and *Fusarium graminearum* Schwabe 110250 (The Westerdijk Fungal Biodiversity Institute, the Netherlands) were used in the experiment. The fungal isolates were cultivated on potato dextrose agar slants (Biolife Srl, Italy) for 7 days at 25°C temperature. The spores were dislodged from the hyphae with a sterile spreader after flooding the culture media with sterile saline solution. The number of spores was determined using the hemocytometer (Feine-Optik, Germany) to a concentration of 10^5 spores ml^{-1}.

Antifungal assay in nutrient medium. The effect of LAB isolates and LAB CFS, separately, on the fungal biomass production in liquid medium was determined by double inoculation in MRS broth. LAB cells were equilibrated according to Grant Instruments™ Liquid McFarland standards (Fisher Scientific Ltd., UK) to 3×10^9 LAB cells ml^{-1}. MRS broth (10 ml) was inoculated with 200 μl of LAB cells or LAB CFS and 200 μl of tested fungal spores. The incubation temperature was 30°C. The fungal growth was determined 7, 14, 21 and 28 days post treatment by extracting fungal biomass on Whatman No. 1 filter paper, which was dried to a constant mass at 50°C temperature for 2 h.

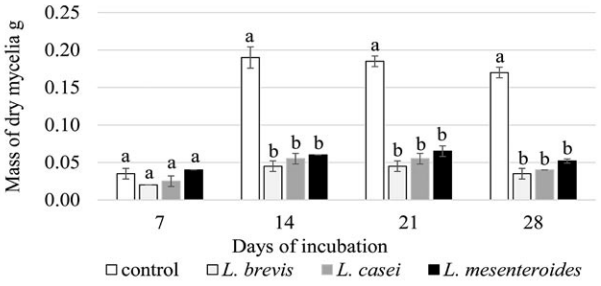
Antifungal assay on wheat grain as a model of natural substrate. Organically cultivated wheat grains (10 g) were placed in a Petri dish and sterilized by pressurized water vapor. Compared to the *in vitro* antifungal assay, where both LAB and LAB CFS were tested, the grains were treated only with LAB CFS according to Deepthi et al. (2016). The fungal isolates (100 μl) were centrally added to the Petri dish containing wheat grains. The fungal growth in two diameters at right angles to each other was evaluated daily until the colony reached the edge of the plate. The mean diameter of each dish was calculated. Fungal growth and time of incubation were used to calculate growth rates. The Petri dishes were incubated in three replicates at 30 ± 0.2°C temperature. The fungal growth and time of incubation were used to calculate growth rates. In the control treatments of both bioassays, sterile distilled water was used instead of LAB. All tests were conducted under laboratory conditions in three replications and repeated twice during 2017 and 2018.

Statistical analysis. The results were statistically analysed by *Statistica*, version 13.5 (TIBCO Software Inc.). All variables were analysed by Student's *t*-test ($P < 0.05$) to test the effects of LAB and compare the differences of fungal growth between the fungal isolates.

Results and discussion

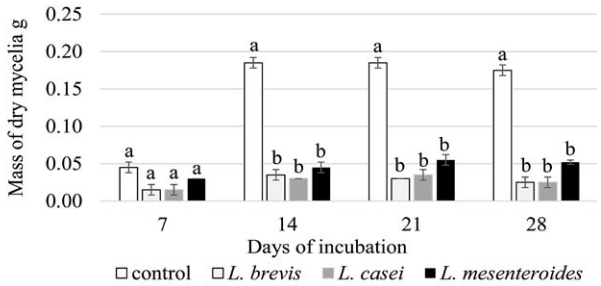
Inhibition of *Penicillium chrysogenum* growth by *Lactobacillus brevis* in culture media ranged from 50% to 79%, *L. casei* inhibited growth from 37% to 76% and *Leuconostoc mesenteroides* reduced growth to 69% (Figure 1). LAB metabolic products in CFS were more effective, where *L. brevis* and *L. casei* inhibited growth

from 66% to 85% and *L. mesenteroides* – from 33% to 75% (Figure 2). Statistical significance was observed between the tested mentioned LAB and the control from 14 to 28 days of incubation ($P < 0.01$).



Note. Different letters in each column group indicate significant differences ($P < 0.05$; Student's *t*-test).

Figure 1. *Penicillium chrysogenum* biomass of dry mycelia affected by lactic acid bacteria (LAB) in culture media

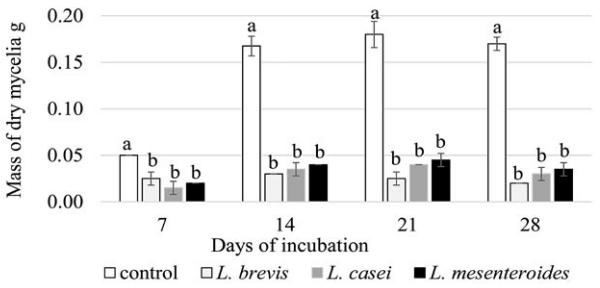


Explanation under Figure 1

Figure 2. *Penicillium chrysogenum* biomass of dry mycelia affected by lactic acid bacteria (LAB) cell-free supernatants (CFS) in culture media

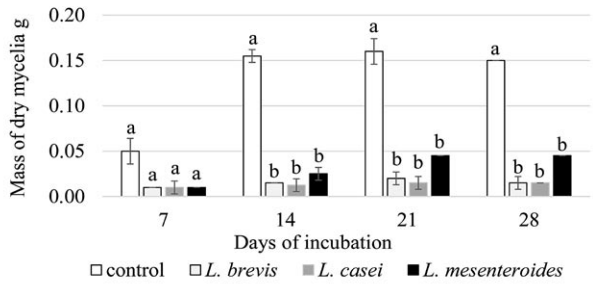
LAB antifungal inhibition of the *Penicillium* spp. has been reported in other studies. Magnusson et al. (2003) have documented that *Pediococcus pentosaceus* inhibited the growth of *Penicillium commune*, and none of the isolates was successful in inhibiting *Penicillium roqueforti* in *in vitro* conditions.

Results of our experiment showed that *Fusarium graminearum* inhibition by *L. brevis* in culture media ranged from 50% to 88%, *L. casei* inhibited growth from 70% to 82% and *L. mesenteroides* – from 60% to 79% (Figure 3). Antifungal products in the CFS were more effective, where inhibition was determined by *L. brevis* from 80% to 90%, by *L. casei* – from 80% to 92% and *L. mesenteroides* – from 70% to 84% (Figure 4). A statistically significant difference of the tested LAB was observed from 7 to 28 days and LAB CFS – from 14 to 28 days of incubation compared to control ($P < 0.01$). Similar to the study of Magnusson et al. (2003), a strong inhibition of the growth of *Fusarium sporotrichoides* by *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *L. coryniformis*, *L. acidophilus*, *L. salivarius*, *L. sakei* and *Pediococcus parvulus* was determined using a dual culture media overlay agar plates. Juodeikiene et al. (2018) found that supernatants of *L. sakei*, *Pediococcus acidilactici*, *P. pentosaceus* KTU05-8, *P. pentosaceus* KTU05-9 and *P. pentosaceus* KTU05-10 are very good inhibitors against *in vitro* mycelial growth and sporulation of *Fusarium culmorum* and *F. poae*. Treatments with the investigated strains resulted in the reduction of zearalenone (ZEA), deoxynivalenol (DON), T-2 and HT-2 toxins concentration in malting grains by 23, 34, 58 and 73 %, respectively. In a study by Franco et al. (2011) commercial culture media Lyofast LPRA (*L. rhamnosus* and *L. plantarum*) showed the greatest inhibition effects against *F. graminearum* IAPAR 2218 using agar diffusion method, also all LAB isolated strains and commercial cultures showed potential for DON removal in liquid media.



Explanation under Figure 1

Figure 3. *Fusarium graminearum* biomass of dry mycelia affected by lactic acid bacteria (LAB) in culture media

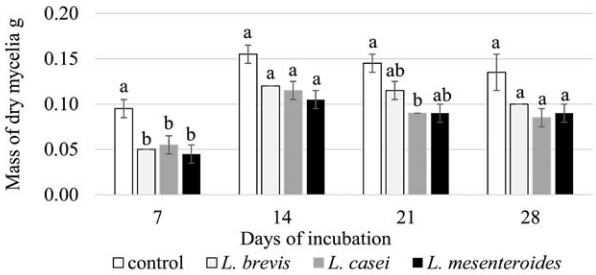


Explanation under Figure 1

Figure 4. *Fusarium graminearum* biomass of dry mycelia affected by lactic acid bacteria (LAB) cell-free supernatants (CFS) in culture media

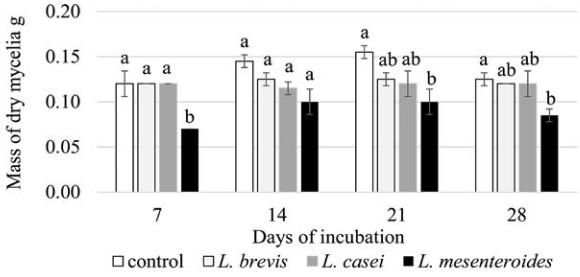
Lactobacillus brevis and *L. casei* showed statistical significance in *Aspergillus* inhibition that decreased with incubation time (Figure 5). CFS *Leuconostoc mesenteroides* showed statistical significance ($P < 0.01$) in the *Aspergillus niger* inhibition ranging from 31% to 42% (Figure 6). The investigated strains of LAB and CFS LAB did not show efficiency determined with previously tested fungi in culture media. In the study of Magnusson et al. (2003), strong growth inhibition of *Aspergillus fumigatus* by the *L. plantarum*, *L. coryniformis* and *P. pentosaceus* was found. Also, in the study of Ben Taheur et al. (2019), *Lactoballus kefiri* in agar medium showed the highest fungal inhibition against *Aspergillus flavus* and *A. carbonarius*. The decrease of fungal biomass was recorded in the study of Kim (2005) in liquid MRS broth inoculated with LAB and *A. fumigatus*: was recorded 75% fungal biomass inhibition in *Lactobacillus curvatus* KC-116, 45% in *Lactococcus lactis* subsp. *lactis* KC-304, 92% in *L. casei* KC-324, 95% in *Lactobacillus pentosus* KC-817, and 40% in *L. sakei* KC-1004, respectively.

The action path of LAB is dependent on various factors: the characteristics of each species or strain of LAB, the properties of the fungi to be inhibit and their biotic interactions. Some species of LAB show specific inhibition patterns, and antifungal compounds identified



Explanation under Figure 1

Figure 5. *Aspergillus niger* biomass of dry mycelia affected by lactic acid bacteria (LAB) in culture media



Explanation under Figure 1

Figure 6. *Aspergillus niger* biomass of dry mycelia affected by lactic acid bacteria (LAB) cell-free supernatants (CFS) in culture media

from LAB CFS are cyclic dipeptides, phenyllactic acid, lactic acid, proteinaceous and some unknown substances (Magnusson, Schnürer, 2001). Yang and Clausen (2005) showed that supernatants of *Lactobacillus casei* subsp. *rhamnosus* and *L. acidophilus* retained antifungal properties in the absence of lactic and phenyllactic acids. In the same study, the investigated LAB CFS produced at least four unknown substances with antifungal properties against *Trichoderma viride* ATCC 20476, *A. niger* 2.242, *P. chrysogenum* PH02 and *Aureobasidium pullulans* MDX-18. These antifungal compounds were heat resistant (to 121°C) and maintained activity after neutralization. Magnusson et al. (2003) have come to a similar conclusion in a study, where the degree of fungal inhibition is not only associated with the production of lactic or acetic acids, but also some antifungal cyclic dipeptides were identified.

In real systems, more complex interactions between the substrate and the antifungal agent prevailed and applied treatments were less effective compared to the first experiment. As well as in an experiment on culture media, none of the LAB species tested statistically inhibited the mycelial growth of *A. niger* on wheat grains (Table).

There was detected a prolongation of incubation time from 4 to 7 days in the treatments when compared to the control, suggesting an antifungal activity but not statistically significant inhibition. There was also no significant inhibition of *P. chrysogenum* on wheat grains, although a decrease in growth was observed in the range of 76–82%, while the incubation time was prolonged from 14 to 16 days compared to the control. A similar influence of the complex interactions between LAB and wheat grains was observed by Suproniene et al. (2015). Antifungal properties of *L. sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7 and *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 on untreated wheat grains were tested. Results showed that the occurrence of *Fusarium* spp. on wheat grains treated with LAB depended on incubation temperature and applied volume of LAB. Strain *P. pentosaceus* and mixtures of *P. acidilactici* + *P. acidilactici* and *L. sakei* + *P. acidilactici* + *P. pentosaceus* significantly reduced growth of *Fusarium* spp., *Bipolaris sorokiniana* and *Alternaria* spp. on wheat grains. Other effects on seed germination and seedling pathogens in laboratory and field trials were insignificant. Researchers concluded that in field conditions LAB failed to produce inhibitory substances for the control of plant pathogens in the complex soil environment.

Table. Mean values of growth rates of the fungal isolates on wheat grains treated with lactic acid bacteria (LAB) cell-free supernatants (CFS)

Tested fungi	*Aspergillus niger*		*Penicillium chrysogenum*		*Fusarium graminearum*	
Treatment, CFS	Growth rate on wheat grains					
	mm day^{-1}	*P*	mm day^{-1}	*P*	mm day^{-1}	*P*
Control	5.4		5.0		7.2	
Lactobacillus brevis	3.5	0.686 ns	1.5	0.963 ns	0.4	<0.01**
Lactobacillus casei	3.4	0.786 ns	1.3	0.594 ns	0.5	<0.01**
Leuconostoc mesenteroides	2.8	0.919 ns	2.4	0.679 ns	0.5	<0.01**

** – growth rate significantly lower than control ($P < 0.01$); ns – not significant

Promising results of this experiment include the antifungal activity of *L. casei*, *L. brevis* and *L. mesenteroides* with 83–90% mean inhibition of *F. graminearum* mycelial growth ($P<0.01$). Similar results were obtained in the study of Abdel-Aziz et al. (2014), where LAB was evaluated against *Fusarium oxysporum*. Tomato seeds were pre-treated with *Lactobacillus* sp., *L. acidophilus* and *L. plantarum* and showed an increase in the root, shoot and seedling lengths as well as vigour index and plant weight. In the study of Varsha et al. (2015), 2,4 DTBP (2,4-di-tert-butyl phenol) obtained from the CFS of *Lactococcus* showed fungicidal activity on wheat grains, where fungal mycelial growth of *F. oxysporum*, *A. niger* and *P. chrysogenum* was completely inhibited. Similar studies of Gupta and Srivastava (2014) have shown that the treatment of wheat grains with peptides of *L. plantarum* LR14 not only prevented the seed borne fungal growth, but the antifungal effect was also retained after 2.5 years of storage under laboratory conditions.

It is evident that the LAB and their metabolites have antifungal activity in culture media and natural substrate and are potential candidates for use in storage conditions. Future research should focus on biotechnical improvement in a real environment. Possible solutions include their application in powder form to prevent increases in grain moisture in storage conditions. Further research also is needed to determine the active antifungal substances that metabolize investigated LAB, to clarify

the complexity of interactions in *in vivo* systems, to examine the effectiveness of treatments on non-sterile grains and to identify interactions between the natural microflora of grains and LAB antifungal substances. Further, it would be desirable to determine the effect of LAB on the biosynthesis of mycotoxins produced by the tested fungi in agricultural systems.

Conclusions

1. The results of this study confirmed the significant antifungal activity of all lactic acid bacteria (LAB) species and their metabolites in the supernatants.

2. *Lactobacillus brevis* and *L. casei* induced significant inhibition of mycelial growth of *Penicillium chrysogenum* and *Fusarium graminearum* in the culture media from 7th to 28th day of incubation. The efficacy of their cell-free supernatants (CFS) was higher compared to the treatments with LAB cells, whereas fungal growth inhibition of *P. chrysogenum* ranged 81–86% and that of *F. graminearum* – 87–92%, respectively.

3. *Leuconostoc mesenteroides* and their CFS were significantly effective in inhibition of all three fungal species and their mycelial growth in the culture media. The best antifungal activity for *Aspergillus niger* was achieved by this LAB species – mean inhibition of fungal growth was 31–50%. The inhibition of fungal growth of *P. chrysogenum* ranged 70–76% and that of *F. graminearum* – 70–92%, respectively.

4. *A. niger* was the most tolerant phytopathogenic fungi in the culture media and natural substrate.

5. On wheat grains as a natural substrate, the LAB treatments were less effective; however, satisfactory results were obtained for *F. graminearum* with 83–90% mean inhibition of mycelial growth.

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Pieno rūgšties bakterijų potencialas *Aspergillus niger, Penicillium chrysogenum* ir *Fusarium graminearum* biokontrolei mitybinėje terpėje ir natūraliame substrate

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Santrauka

Nepalankios sandėliavimo sąlygos blogina sandėliuojamų grūdų kokybę ir skatina grybų augimą bei dauginimąsi. Grūdų kokybę gali padėti išsaugoti priešgrybiniai biopreparatai. Sandėliavimo metu arba siekiant nukenksminti sandėliuojamus produktus potencialiai gali būti naudojami pieno rūgšties bakterijos (PRB) ir priešgrybinių savybių turintys jų metabolitai. Tyrimo tikslas – nustatyti *Lactobacillus casei*, *Lactobacillus brevis* ir *Leuconostoc mesenteroides* priešgrybinį poveikį patogeninių grybų *Aspergillus niger*, *Penicillium chrysogenum* ir *Fusarium graminearum* grybienos augimo slopinimui. PRB biokontrolės priešgrybinis potencialas buvo tirtas laboratorinėmis sąlygomis mitybinėse terpėse, kurios yra idealūs grybų augimo substratai, ir kviečių grūduose, tyrimo metu naudotų kaip natūralus substratas. Skysta terpė (SKT/MRS sultinys) buvo inokuliuota visų rūšių PRB arba PRB supernatantais ir grybų sporomis. Grybų augimas buvo įvertintas matuojant grybienos biomasės padidėjimą po inkubacijos praėjus 7, 14, 21 ir 28 dienoms. Antras eksperimentas buvo atliktas su kviečių grūdais, apdorotais PRB supernatantais ir užkrėstais grybų sporomis. Biopreparatas, kuriame yra visų rūšių PRB arba jų supernatantų, reikšmingai slopino grybų augimą *P. chrysogenum* (slopino vidutiniškai 69–75 % PRB ir 80–81 % PRB supernatantų) ir *F. graminearum* (slopino vidutiniškai 60–83 % PRB ir 83–88 % PRB supernatantų) mitybinėse terpėse. Eksperimente su kviečių grūdais visų tirtų rūšių PRB supernatantai reikšmingai (vidutiniškai 83–90 %) slopino tik *F. graminearum* augimą.

Lyginant eksperimentų rezultatus buvo pastebėtas PRB preparatų efektyvumo nenuoseklumas. Rezultatai rodo, kad per eksperimentą naudotos terpės darė įtaką tirtų bakterijų ir grybų aktyvumui bei tolerancijai. *L. casei*, *L. brev*is ir *L. mesenteroides* priešgrybinės savybės buvo nustatytos mitybinėje terpėje ir natūraliame substrate. *F. graminearum* buvo jautriausias, o *A. niger* tolerantiškiausias apdorojimui PRB ir PRB supernatantais.

Siekiant PRB panaudoti sandėliuojant grūdus, reikia atlikti daugiau tyrimų, kurie atskleistų jų poveikį fitopatogeniniams grybams skirtingomis sąlygomis.

Reikšminiai žodžiai: *Lactobacillus brevis*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, kviečių grūdai, priešgrybinis aktyvumas.