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Antibiotic-resistant mutants of lactic acid bacteria: potential food control agents

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ABSTRACT

Background: One of the most pressing challenges facing global agriculture today is the restoration of degraded farmland. Organic fertilisers are increasingly being used as a safer alternative to synthetic forms. The most promising form of environmentally friendly fertiliser is a bioconsortium based on different groups of microorganisms. Lactic acid bacteria (LAB) with high antifungal activity, isolated from Armenian dairy products, can form part of such a bioconsortium and protect plants from phytopathogens. In addition, LAB can be used as a preservative in the food industry due to its GRAS (generally recognised as safe) status.

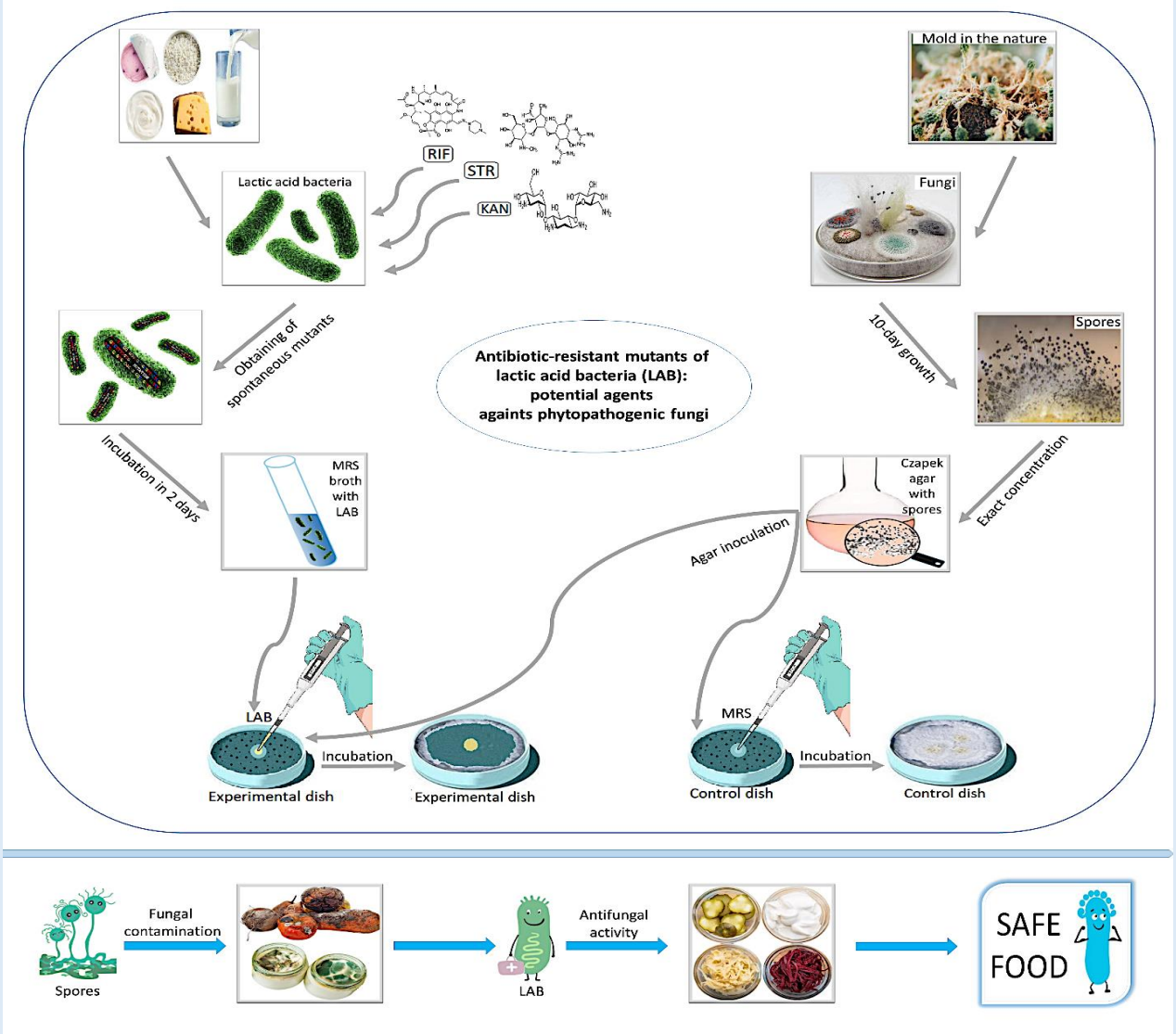
Objective: To obtain antibiotic-resistant pleiotropic lactic acid bacterial mutants with higher antifungal activity and broad-spectrum activity compared to natural strains.

Methods: The objects of the study were LAB, whose antifungal activity was studied against phytopathogenic fungi of the genera *Penicillium*, *Aspergillus*, *Alternaria* and *Fusarium*, which cause crop/food spoilage. To increase antifungal activity, spontaneous pleiotropic mutants resistant to the streptomycin, kanamycin or rifampicin were obtained on the basis of original strains.

Results: Among all the LAB tested, *Lactobacillus bovis* MDC 1061, *L. paracasei* MDC 10898 and *L. buchneri* BKM 1599 were selected for their antifungal activity. Based on the latter, 78 antibiotic-resistant mutants with different levels of antifungal activity were obtained. L. bovis MDC 1061 STR-25 and L. buchneri BKM 1599 RIF-7 mutants with up to 98% and 95% inhibition of fungal growth, respectively, were selected as potential food control agents.

Conclusion: Antibiotic-resistant mutants L. *bovis* MDC 1061 STR-25 and L. *buchneri* BKM 1599 RIF-7 with high antifungal ability and a wide range of its effects were obtained. For the first time, the phenomenon of pleiotropic effect has been used in LAB to improve antifungal activity. After extensive study, these mutants can be used as promising components for creating an effective bioconsortium, as well as a preservative in food production, especially in the case of plant-based functional foods to protect against contamination by fungi.

Keywords: lactic acid bacteria, pleiotropic mutants, antifungal activity



INTRODUCTION

Nowadays, the use of environmentally friendly fertilizers is very important in global agriculture. Many adverse environmental changes, such as soil compaction, salinization, pollution with toxic wastes, etc. caused by chemical fertilizers can be avoided by using environmentally friendly products in agriculture. Such products, even in large quantities, do not have negative consequences, but rather restore degraded arable soils and increase their fertility by stimulating the growth and development of agricultural plants, as well as serve as an effective biocontrol tool against a wide range of fungal and bacterial phytopathogens [1-4].

Widespread mold fungal infection is one of the most unfavorable factors for plant growth, triggering various plant diseases, and causing significant damage to agriculture. The process of infection triggers production of mycotoxins - highly toxic fungal metabolites, with carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic, and hepatotoxic effects. Fungal contamination can occur at any stage of the food chain (during harvesting, processing and storage, etc.): airborne and through infected clothing [5-7].

One of the modern methods used to suppress the process of fungal infection of plants is the application of compounds with antifungal activity, such as chemical fungicides and pesticides. However, this practice can lead to environmental pollution and pose risks to human and animal health [8, 9]. Due to their chemical origin, these fungicides affect the functional status of nutrients containing food bioactive compounds (FBCs). It's very important to prevent this because functional foods provide health benefits by reducing the risk of chronic diseases [10, 11].

Currently, scientists working in the field of agriculture are increasingly concerned with the phenomenon of antagonism between certain microorganisms. This phenomenon, with the use of "biological control agents" (yeast and bacterial antagonists), is recognized as one of the most relevant and effective in the control of phytopathogenic microorganisms [12-14].

From this point of view, lactic acid bacteria isolated from natural sources are of particular interest. They have been widely used by humans for centuries due to their harmlessness and their GRAS status. They are characterized by high antimicrobial activity due to the fact, that they synthesize a wide range of compounds, such as diketopiperazine, 3-phenyllactate, hydrogen peroxide, diacetyl, etc., and are considered as promising natural antagonists of phytopathogenic fungi and bacteria [15-18]. Because of these properties, lactic acid bacteria are widely used in various fields, such as in the food industry as preservatives, in agriculture as part of biofertilizers, and in medicine as probiotics [18, 19, 20].

Bioconsortium - communities of microorganisms that can effectively perform their assigned tasks, especially, stimulate the absorption of nutrients from the environment and increase plant resistance to various adverse factors, are the most productive option for biofertilizers. One of these microorganisms may be LAB because of its function in protecting plants from phytopathogens [21-23].

Effective LAB strains with a wide spectrum of antifungal activity can be obtained on the basis of cultures with antifungal activity already present in nature, using the known phenomenon of pleiotropic effect observed in antibiotic-resistant mutants with altered functioning of the protein-synthesizing apparatus [24-26].

The aim of the study is to obtain LAB mutants with higher antifungal activity and a wider range of effects on molds compared to the original strains.

MATERIALS AND METHODS

Strains: The objects of this study were 17 strains of lactic acid bacteria and 12 strains of fungi (Table 1). All these strains, both bacteria and fungi, were provided by the Microbial Depository Center of the Scientific and Production Center ''Armbiotechnology'' of the National Academy of Sciences of Armenia.

Table 1. Strains of lactic acid bacteria and microscopic fungi studied in the work

№	Lactic acid bacteria	
1	*Lactobacillus*	*crispatus* MDC 9612
2		*brevis* NCDO 477
3		*delbrueckii* BKM 1596
4		*rhamnosus* BKM 574
5		*casei* NCDO 161
6		*plantarum* NCDO 820
7		*helveticus* MDC 3722
8		*bulgaricus* MDC 507
9		*bovis* MDC 1061
10		*acidophilus* BKM 1660
11		*paracasei* MDC 10898
12		*buchneri* BKM 1599
13		*pentosus* MDC 9625
14		*lactis* MDC 10881
15		*fermentum* NCDO 215
16	*Streptococcus*	*thermophilus* MDC 9608
17		*lactis* MDC 9615

№	Microscopic fungi	
1	*Penicillium*	*funiculosum* MDC 8258
2		*luteum* MDC 8072
3		*commune* MDC 8299
4		*commune* MDC 8313
5		*chrysogenum* MDC 8305
6		*chrysogenum* MDC 8366
7	*Fusarium*	*moniliforme* MDC 8127
8		*oxysporum* MDC 8385
9	*Aspergillus*	*puniceus* MDC 8255
10		*fumigatus* MDC 8344
11		*niger* MDC 8449
12	*Alternaria*	*alternate* MDC 8180

Growth medium: Lactic acid bacteria were maintained in skim milk medium: powdered milk – 130.0 g, distilled water – 1000 ml, pH 6.6, autoclaved at 121 °C for 10 min. Milk powder from Katnarat factory (Katnarat, Armenia) was used for the preparation of the medium.

For antifungal activity screening, LAB strains were grown in De Man, Rogosa and Sharpe (MRS) broth of the following composition: yeast extract – 5.0 g, beef extract – 10.0 g, peptone – 10.0 g, dextrose – 20.0 g, Tween 80 – 1.0 g, ammonium citrate – 2.0 g, manganese sulfate – 0.05 g, distilled water – 1000 ml, pH 6.5, autoclaved at 121 °C for 15 min. Bacteriological agar (12.0 g/l) was added to the composition of the broth to prepare the solid medium.

Fungi were grown on Czapek Dox agar: sucrose – 30.0 g, sodium nitrate – 2.0 g, dipotassium phosphate – 1.0 g, magnesium sulfate – 0.5 g, potassium chloride – 0.5 g, ferrous sulfate – 0.01 g, agar – 15.0 g, distilled water – 1000 ml, pH 6.5. The medium was autoclaved at 121 °C for 15 min.

Study of the antifungal activity of LAB: LAB was grown in MRS broth in 2 days on the New Brunswick Innova 43 incubator shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and 37 °C. The 2-day growth was necessary not only to achieve 10^8-10^9 CFU/ml of viable cells, but also to reach the highest inhibitory effect [2 7].

Spores of 10-day grown fungi, collected by inoculation loop and transferred to physiological solution, were used for the experiment. The concentration of spores in the solution was counted by using the Malassez hemocytometer (Thermo Fisher Scientific, Waltham, USA).

The antifungal activity of LAB was studied in Czapek-Dox agar medium. A spore suspension was added to Czapek-Dox medium (with a final

concentration of 10^2 or 10^4 or 10^8 spores/ml: depending on the experiment) and poured into Petri dishes (10 cm in diameter) in 20 ml. A hole (0.5 cm diameter) was then made in the center of each dish and 200 µL of LAB suspension was added. For the control plate, pure MRS broth was added to the hole. The experimental plates were incubated at 28 °C for 10 days. Antifungal activity was determined by measuring the diameter of the zone of inhibition using calipers.

Obtaining antibiotic-resistant spontaneous mutants of LAB: To increase the antifungal activity of selected LAB, spontaneous mutants resistant to streptomycin (STR), kanamycin (KAN) or rifampicin (RIF) were obtained. First, the minimum inhibitory concentration of antibiotics was determined, using 0-70 µg/ml RIF (with a unit range of 5 µg/ml) and 0-350 µg/ml KAN/STR (with a unit range of 50 µg/ml). The LAB overnight suspension (to a final concentration of 10^8-10^9 CFU/ml) was plated on MRS agar and cultured at 37°C for 24 hours to form a bacterial lawn. An antibiotic disc impregnated with a specific concentration of antibiotic solution was then placed in the centre of the dish and Petri dishes were incubated for 48 hours. The inhibitory concentration of antibiotics was determined by measuring the diameter of the inhibition zone with a caliper. The minimum inhibitory concentration of the antibiotic was chosen as the dose at which there was no bacterial growth.

To obtain antibiotic-resistant spontaneous mutants, the overnight bacterial suspension was plated on MRS agar containing the minimum inhibitory concentration of antibiotic. Dishes were incubated for 5-6 days as the growth of bacterial cells is slowed by the action of antibiotics. The antibiotic resistance of all grown colonies was then tested by growing them again on the medium containing the minimum inhibitory concentration of antibiotic. Colonies that grew a second time were classified as antibiotic-resistant mutants and were used for further work.

Statistical Analysis: All antifungal activity experiments were performed in triplicate and data analysis was performed by ANOVA with Dunnett's test using Minitab 17.1 statistical program (Minitab Inc., Pennsylvania, USA).

RESULTS

Lactic acid bacteria can serve as an important component of the bacterial consortium. To select strains with antagonistic activity, we evaluated the antifungal ability of LAB of the genera *Lactobacillus* and *Streptococcus* against molds of the genera *Penicillium*, *Aspergillus*, *Alternaria*, and *Fusarium* (Table 1). The choice of fungal strains for testing was based on the fact that representatives of these particular genera are widespread in nature, especially in soil and cause crop/food spoilage [2 8, 2 9].

The antifungal activity of LAB strains was tested in two steps. In the first step, a screening of lactic acid bacteria was performed using fungi at a concentration of 10^2 spores/ml. For the next stage, eight of the strains studied were selected that had high antifungal activity and were able to inhibit the growth of two or more of the fungi tested (Figure 1).

In the second stage, the mold was used in a higher concentration (10^4 spores/ml) compared to the first stage. Figure 2 show that the strains *L. bovis* MDC 1061, *L. paracasei* MDC 10898 and *L. buchneri* BKM 1599 have the highest antagonistic activity against the test fungi. In addition, the same strains were characterized by a broader spectrum of antifungal activity.

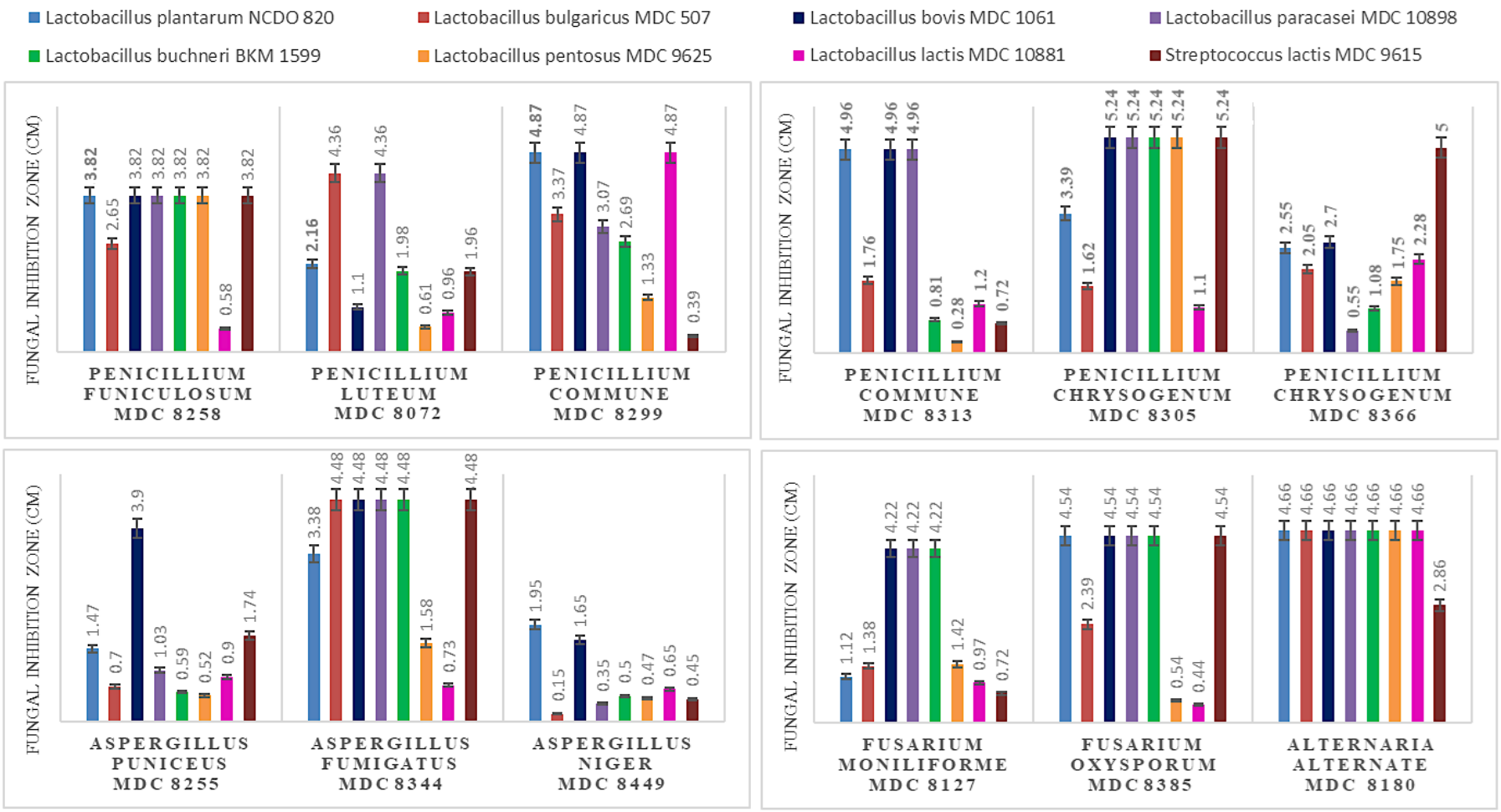


Figure 1. Screening of lactic acid bacteria for antifungal activity (at a fungal concentration of 10^2 spores/ml)

Fungal growth control: P. funiculosum MDC 8258 – 3.82 cm, P. luteum MDC 8072 – 4.36 cm, P. commune MDC 8299 – 4.87 cm, P. commune MDC 8313 – 4.96 cm, P. chrysogenum MDC 8305 – 5.24 cm, P. chrysogenum MDC 8366 – 5.00 cm, A. puniceus MDC 8255 – 3.90 cm, A. fumigatus MDC 8344 – 4,48 cm, A. niger MDC 8449 – 5.15 cm, F. moniliforme MDC 8127 – 4.22 cm, F. oxysporum MDC 8385 – 4.54 cm, Al. alternate MDC 8180 – 4.66 cm ($p<0.01$)

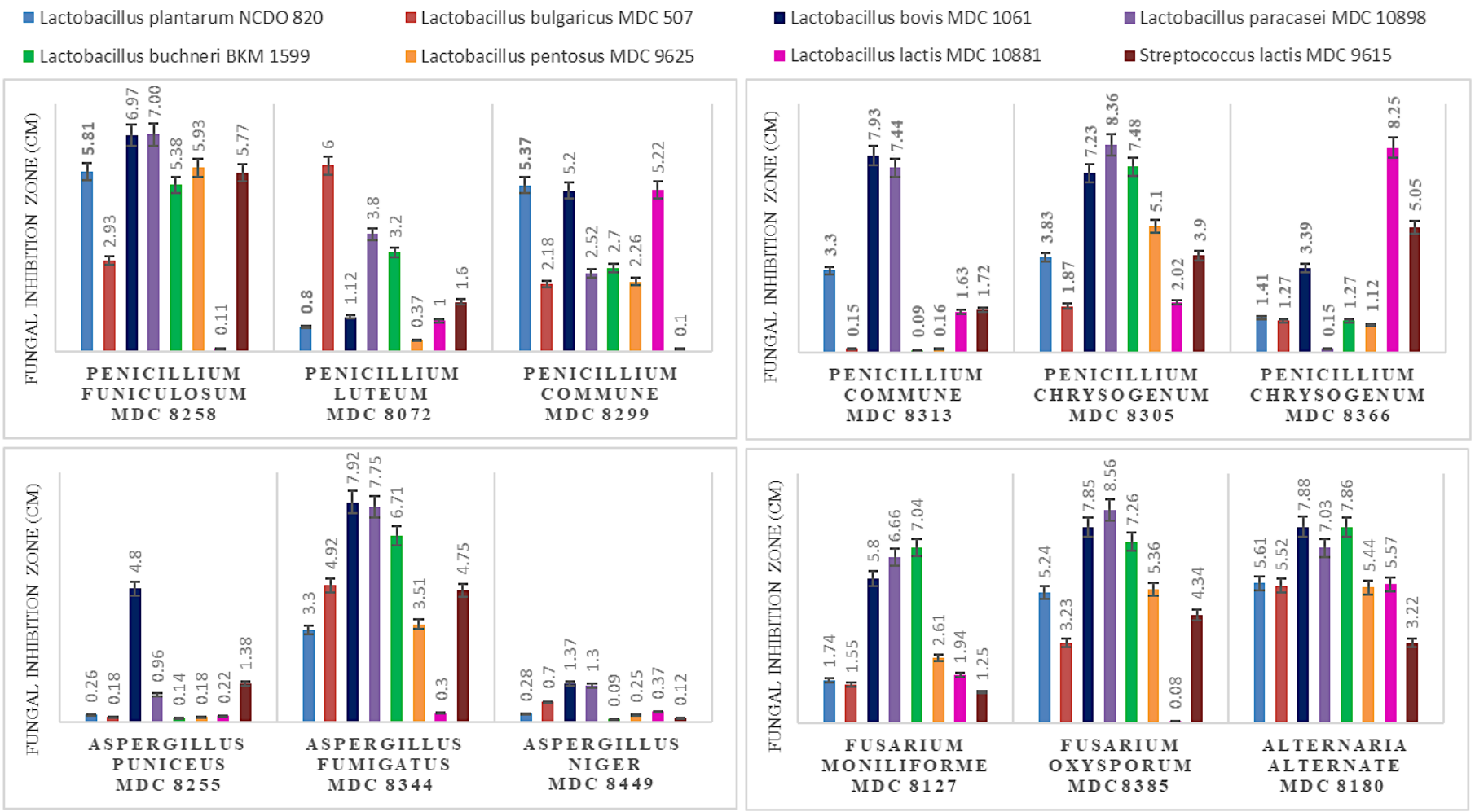


Figure 2. Screening of lactic acid bacteria for antifungal activity (at a fungal concentration of 10^4 spores/ml)

Fungal growth control: *P. funiculosum* MDC 8258 – 7.25 cm, *P. luteum* MDC 8072 – 8.00 cm, *P. commune* MDC 8299 – 8.64 cm, *P. commune* MDC 8313 – 8.25 cm, *P. chrysogenum* MDC 8305 – 8.50 cm, *P. chrysogenum* MDC 8366 – 8.37 cm, *A. puniceus* MDC 8255 – 6.8 cm, *A. fumigatus* MDC 8344 – 8.16 cm, *A. niger* MDC 8449 – 8.25 cm, *F. moniliforme* MDC 8127 – 8.42 cm, *F. oxysporum* MDC 8385 – 8.72 cm, *Al. alternate* MDC 8180 – 7.98 cm (*p<0.*

To create a bioconsortium, LAB strains with higher antifungal activity compared to the initial cultures, and at the same time with a wide spectrum of this activity, were needed. The required LAB strains were obtained by exploiting the well-known pleiotropic effect of some spontaneous mutations resistant to certain antibiotics, in particular streptomycin, kanamycin and rifampicin. The minimum inhibitory concentrations of antibiotics to obtain such mutants were determined experimentally: 300 µg/ml for STR and KAN and 65 µg/ml for RIF (Table 2). Seventy-eight antibiotic-resistant LAB mutants were obtained: 16 mutants in *L. bovis* 1061 (Rif-r – 6, Kan-r – 8, St-r – 13), 26 mutants in *L. paracasei* MDC 10898 (Rif-r – 7, Kan-r – 6, St-r – 11), and 21 mutants in *L. buchneri* BKM 1599 (Rif-r – 13, Kan-r – 4, St-r – 10).

Table 2. Determination of antibiotic inhibitory concentration.

Antibiotics		**Lactic acid bacteria**		
		Bacterial growth		
	Concentration (µg/ml)	*Lactobacillus bovi* MDC 1061	*Lactobacillus paracasei* 10898	*Lactobacillus buchne* BKM 1599
Rifampicin	0	++++	++++	++++
	30	++++	+++	+++
	45	++	++	++
	60	±	+	±
	65	-	-	-
	70	-	-	-
Kanamycin	0	++++	++++	++++
	200	+++	+++	+
	250	++	++	±
	300	-	-	-
	350	-	-	-
Streptomycin	0	++++	++++	++++
	200	++	+++	++
	250	±	+	+
	300	-	-	-
	350	-	-	-

(++++) - no inhibition zone, (+++) - inhibition zone on 5-25% of the agar diameter, (++) - inhibition zone on 25-50%, (+) - inhibition zone on 50-75%, (±) - inhibition zone on 75-95%, (-) - no bacterial growth

Figure 3 shows the antifungal activity of LAB mutants, which, as we expected, show changes in antifungal activity compared to the original strains. In particular, mutants Str 19, Str 25 of *L. bovis* 1061 and Rif 7, Rif 14, Rif 21 of *L. buchneri* BKM 1599 cause 80-90% growth suppression on average in most of the fungi tested ($p<0.01$). The data show that in some of the mutants a significant decrease in antifungal activity is observed, while in the Kan 7 mutant of *L bovis* strain 1061 (in the case of fungi *F. moniliforme* 8127), Rif 13 mutant of *L. paracasei* MDC 10898 (in the case of *F. oxysporum* 8385) and Rif 14 mutant of *L. buchneri* BKM 1599 (in the case of *P. chrysogenum* MDC 8305) this ability can be considered as lost. The remaining mutants, which are not shown in the table, have only a low ability to suppress the growth of molds. In order to confirm the high antifungal activity of the selected mutants, they were studied in experiments with test fungi in higher concentration than before (10^8 spores/ml instead of 10^4 spores/ml) (Figure 4). Based on the results, it can be concluded that among the obtained antibiotic-resistant mutants, Str 25 of strain *L. bovis* 1061 and Rif 7 of strain *L. buchneri* BKM 1599 are the most promising, since these mutants have a pronounced antifungal ability and at the same time a wide range of its effects.

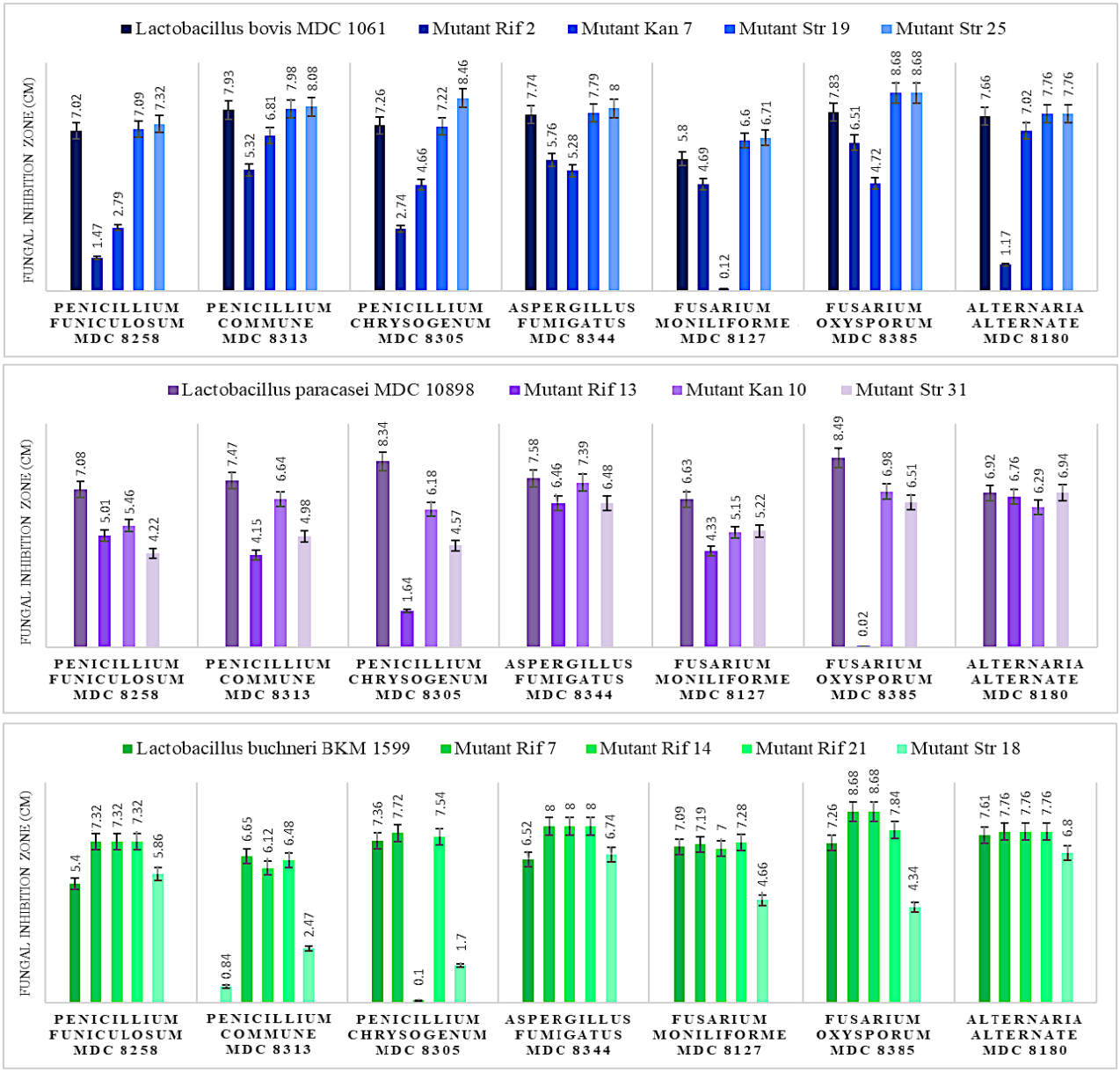


Figure 3. Comparative antifungal activity of the original LAB strains and their pleiotropic mutants (at a fungal concentration of 10^4 spores/ml) Fungal growth control: *P. funiculosum* MDC 8258 – 7.32 cm, *P. commune* MDC 8313 – 8.3 cm, *P. chrysogenum* MDC 8305 – 8.46 cm, *A. fumigatus* MDC 8344 – 8.0 cm, *F. moniliforme* MDC 8127 – 7.46 cm, *F. oxysporum* MDC 8385 – 8.68 cm, *Al. alternate* MDC 8180 – 7.76 cm ($p<0.01$)

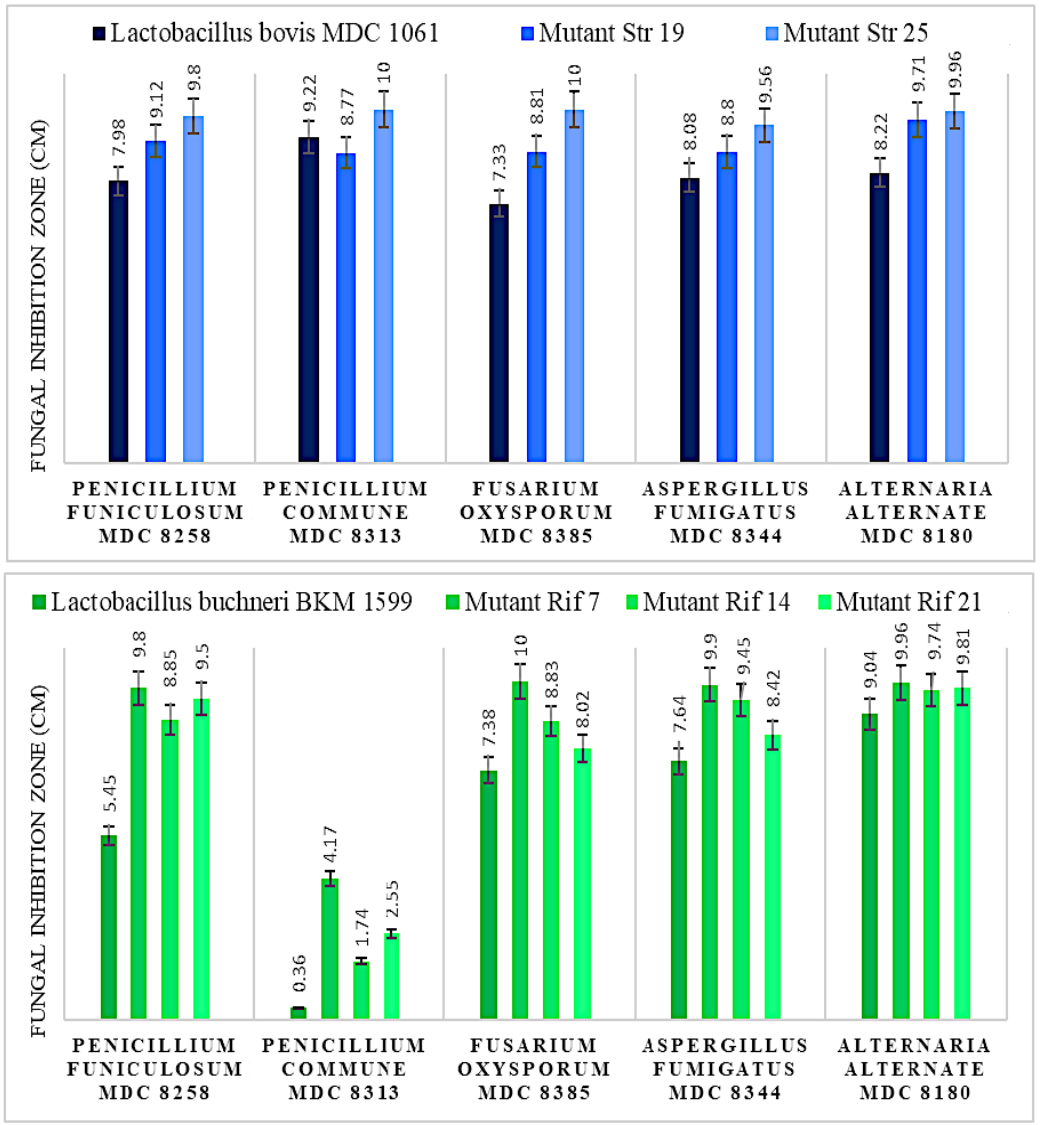


Figure 4. Comparative antifungal activity of the original LAB strains and their pleiotropic mutants (at a fungal concentration of 10^8 spores/ml) Fungal growth control: *P. funiculosum* MDC 8258 – 9.1 cm, *P. commune* MDC 8313 – 10.0 cm, *F. oxysporum* MDC 8385 – 10.0 cm, *A. fumigatus* MDC 8344 – 9.8 cm, *Al. alternate* MDC 8180 – 9.96 cm ($p<0.01$

DISCUSSION

The world's rapidly growing population is expected to reach around 10 billion by 2050. It appears that food production will have to increase many times over in the near future, which is unrealistic given the current economic losses of 20-40% per year due to depletion of natural resources, climate change, prolonged use of chemical fertilisers, plant diseases caused by phytopathogens and pests [6, 30 , 31].

Under such alarming conditions, it is particularly important to prevent the loss of plant-based functional foods with FBCs that are functionally important for human health: associated with reduced risk of many chronic diseases, including heart disease, cancer, and diabetes [32, 33].

Agrochemicals can be replaced by the less dangerous biological preparations - bioconsortiums, which consist of a community of microorganisms with a number of valuable properties that contribute to the growth and development of agricultural crops [2 2, 3 4].

In order to obtain strains for future use within the bioconsortium, the first step was to screen the LAB provided by the Microbial Depository Center of the SPC "Armbiotechnology" NAS RA. All these bacteria were isolated from traditional Armenian dairy products (Table 1). The initial screening (at a fungal concentration of 10^2 spores/ml) showed that only 8 strains of LAB were able to completely inhibit the growth of two or more fungi tested. Figure 1 shows that *Alternaria alternate* MDC 8180 was the most inhibitory fungus (mean 95%, *p<0.01*), whereas *Aspergillus niger* MDC 8449 was significantly inhibited only by *L. plantarum* NCDO 820 and *L. bovis* MDC 1061 (38% and 32% respectively, *p<0.01*).

Figure 2 shows that at a spore concentration of 104 spores/ml, 3 strains have high antifungal activity: L. bovis MDC 1061 against 9 fungi (average 85%, p<0.01), L. paracasei MDC 10898 against 8 fungi (average 87%, p<0.01) and L. buchneri BKM 1599 against 6 fungi (average 84%, p<0.01).

In order to increase antifungal activity, seventy-eight spontaneous pleiotropic mutants with impaired protein synthesis at the level of transcription or translation of genetic information have been obtained on the basis of the above three LAB strains. Such mutations can cause its divergence at the level of virtually any gene, which in turn can lead to simultaneous changes in a number of properties of the mutant cell, such as morphology, growth rate, synthesis of secondary metabolites, improvement or deterioration of technological and many other characteristics, etc., so its may also be responsible for an increase in antifungal activity [2 4-2 6].

As expected, the antibiotic-resistant mutants based on the same original strain show different levels of antifungal activity as a result of pleiotropic mutations. Figure 3 shows the antifungal abilities of the more active mutants, which are able to suppress the growth of most of the fungi tested. In the case of *L. bovis* MDC 1061 (with 90% suppression of fungal growth on average, *p<0.01*), the Str 19 and Str 25 mutants showed increased activity: 95% and 98% respectively. However, the antifungal activity of the Rif 2 and Kan 7 mutants decreased: 50% and 55% on average respectively, in addition Kan 7 showed only trivial activity (1-2%) against the fungus *F. moniliforme* MDC 8127. The mutants of *L. paracasei* MDC 10898 (with 94% suppression of fungal growth on average, *p<0.01*) showed reduced activity against all fungi tested (about 50-70% on average), and in the case of the Rif 13 mutant a complete loss of activity against *F. oxysporum* MDC 8385 was observed. The Rif 7, Rif 14 and Rif 21 mutants of *L. buchneri* BKM 1599 (with 75% suppression of fungal growth n average, *p<0.01*) showed better results: 95%, 82% and 93% respectively,

and and the Rif 14 mutant only shows very low activity (up to 1%) in the case of *P. chrysogenum* MDC 8305. Moreover, in the case of *P. commune* MDC 8313, these three mutants showed 7-8 times higher activity, for example, the antifungal ability of Rif 7 was 80% against 10% of the original strain.

The next experiments to test the antifungal activity of the most promising mutants showed that the growth inhibition of five tested fungi continued even at higher spore concentrations: 10^8 spores/ml (Figure 4). Although the antifungal activity of the Str19 mutant of *L. bovis* MDC 1061 (with an average of 83% suppression of fungal growth, $p<0.01$) was lower than that of the original strain in the case of *P. commune* MDC 8313, Str 25 showed consistently higher activity: 98% on average for all five fungi. Among the mutants of *L. bovis* MDC 1061 (with an average of 60% suppression of fungal growth, $p<0.01$), RIF-7 has the highest activity with 95% suppression, inhibiting the growth of all five fungi. In the case of *P. commune* MDC 8313, the inhibition of fungal growth is lower than at the time of 104 spores/ml concentration, but the mutants again showed many times greater activity (about 10 times) compared to the original strain with 4% inhibition, and the most active was Rif 7 with 40% suppression.

As a result, the most promising antagonists were the *L. bovis* 1061 Str 25 and *L. buchneri* BKM 1599 Rif 7, which can be recommended for use in the development of an effective multifunctional biofertiliser after a detailed study of their viability in soil as well as their competitiveness and compatibility with the beneficial soil microflora.

CONCLUSION

The work demonstrates the feasibility of using the effect of pleiotropic mutations in the synthesis of secondary metabolites as a tool for solving practical problems. It is the first time this effect is used in LAB to improve antifungal properties. The resulting mutants, STR-25 from *L. bovis* strain MDC 1061 and RIF-7 from *L. buchneri* strain VKM 1599, inhibited fungal growth by up to 98% and 95%, respectively, and may serve as promising components for the creation of an effective bacterial consortium and as potential friendly food control agents.

Future research of these obtained LAB will be their use as part of a bioconsortium, where their main function will be to protect plants from phytopathogens. The second group of the bacteria in bioconsortium will be osmoresistant nitrogen-fixing bacteria previously isolated by us from saline soils in Armenia to provide plants with necessary nutrients [3 5].

List of Abbreviation: LAB: lactic acid bacteria, GRAS: generally recognized as safe, FBC: food bioactive compounds, MRS: De Man, Rogosa and Sharpe, Str: streptomycin, Kan: kanamycin, Rif: rifampicin, ANOVA: Analysis of variance, FAO: Food and Agriculture Organization.

Competing interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions: SK: supervision, methodology, writing – original draft preparation, data curation. ZhK: investigation, methodology, writing – review and editing, data curation. AT: investigation, methodology, writing – original draft preparation, writing – review and editing. GA and LM: supervision, writing – review and editing. NKh: investigation, methodology, writing – review and editing. SG: methodology, data curation. KCh: methodology, writing – review and editing. VGh:

supervision, data curation. GTs: investigation, data curation

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