ANTAGONISTIC ACTIVITY OF *Lactococcus lactis* ssp. AND *Streptococcus thermophilus* AGAINST *Bacillus* AND *Pseudomonas* SPECIES

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Abstract: Lactic acid bacteria (LAB) are one of the most important groups of bacteria in the food industry, and are able to inhibit the growth of spoilage or pathogenic microorganisms due to the biosynthesis of various compounds with antibacterial activity. The study included 5 strains of subspecies *Lactococcus lactis,* 2 strains *Streptococcus thermophilus* and 11 bacterial strains of *Bacillus* and *Pseudomonas* genera stored and deposited in National Collection of Non-pathogenic Microorganisms (NCNM) of Republic of Moldova. Antagonistic activity was determined using agar well diffusion method. The obtained results had shown that after long-term storage by lyophilization autochthonous strains demonstrated viability and stability of morphological, cultural and technological characteristics such as acidification and coagulation, which are important for dairy industry. According to the present study LAB possesses inhibitory effect which varied significantly depending on strains and cultivation media used. It was observed that some strains are potential candidates for development of microbial preparations against food spoilage causing bacteria such as *Bacillus* spp. and *Pseudomonas fluorescens*. The research suggests to the need to selection of nutrient components that will allow to enhance strain-specific properties of studied strains of biotechnological interest and to expand the directions of their implementation.

Key words: antibacterial activity; inhibition zone; *Pseudomonas*; *Bacillus*; lactic acid bacteria.

INTRODUCTION

Representatives of *Lactococcus* and *Streptococcus* lactic acid bacteria (LAB) isolated from naturally fermented homemade dairy products and stored in the NCNM can be involved in educational, research, industrial application, directed to economic problems solution such as pure cultures application in food and dairy industry [4]. *Lactococcus lactis* ssp. *lactis* are destinated for application as active acidifier for production of sour cream, fresh cheese, brined cheese. *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* contribute to typical flavor and aroma of cheeses due to the production of diacetyl (Camembert, Cheddar, Emmental cheeses). *Streptococcus thermophilus* is lactic acid strain combined with the *Lactobacillus delbrueckii* for yogurt obtaining, flavour and texture, produce exopolysaccharides. *Lactococcus lactis* ssp. *cremoris* strains used to prevent active acid formation in manufacture of cream, sour milk, fresh cheese [4, 19, 24].

LAB are one of the most important groups of bacteria in the food industry, Gram-positive, catalase-negative, facultative anaerobic, nonmotile, and non-spore-forming bacteria, have spherical cells and at morphology occur singly or in chains. In the composition of starter cultures for dairy products are used rod - shape *Lactobacillus* strains and spherical *Lactococcus, Streptococcus, Pediococcus* and *Leuconostoc* strains, but during manufacturing process and storing may appear potentially dangerous microorganisms [7, 30]. Microbial contamination of milk products can be related to non-compliance with hygiene, technological requirements, contaminated equipment, water sources, additives. LAB with antibacterial properties can prevent microbial contamination, due to inhibitory substances such as bacteriocins, diacetyl, organic acids, which contribute to the product bioprotection. These antimicrobial components are safe bioconservatives in great demand in the food industry. Therefore, agricultural biotechnology significantly expanded and improved the directions of implementation of these microorganisms. LAB are applied in sustainable agriculture in biological control of bacterial plant diseases, for plant growth-promoting effects, as fertilizers for biodegradation [23].

Bacteriocin produced by the *L. lactis* strain demonstrates an increased antimicrobial activity against *Escherichia coli, Streptococcus agalactiae, Micrococcus luteus, Staphylococcus xylosus, Candida albicans, Enterococcus faecium* [25], and bacteriocin of *S. thermophilus* active against *Bacillus cereus, Bacillus subtilis, Listeria innocua, Enterococcus faecalis, Staphylococcus epidermidis* [19]. Fusieger *et al.* [8] showed that *L. lactis* subsp. *lactis* bv. *diacetylactis* strain with inhibitory bacteriocinogenic potential, due to its ability to produce nisin, becomes a promising candidate as a starter culture with biopreservative potential.

Food spoilage is mainly associated with pathogenic microorganisms, but, according to the new data, the problem of food spoilage caused by non-pathogenic *Bacillus subtilis* and *Pseudomonas fluorescens* continues to attract increasing attention from researchers [15, 17, 20]. *P. fluorescens* is Gram-negative, non-spores former bacteria with wide range of growth temperature, causes food spoilage by enzymes production of refrigerated food items, in packed ready to-eat vegetables, in stored chicken meat, fish, beef, cheese, milk due to protease, lecithinase, and lipase activity [15]. Compared to Gram-negative *P. fluorescens*, *Bacillus* species are Gram-positive, spore-former bacteria, presents in food processing lines, are involved in milk, dairy and meat products spoilage, can reach the products in each phase of storage and

ripening [16, 20, 29]. Thus, screening of microorganisms and development of safe and effective microbial preparations for food biotechnology represents actual research.

The stability of technological properties is a fundamental requirement for biotechnology relevant bacterial strains, considering that technological processes can cause cell damage, and consequently to loss of viability and biological activity [5]. Therefore maintaining the viability of deposited strains and their technological properties is an important task for every ex-situ microbial collection. Smith and Ryan [26] highlighted the necessity to offer high quality biomaterial with features defined in passports, and importance of quality management work respecting international criteria.

The aim of the present study was to evaluate the antibacterial activity and to determine the zone of inhibition of certain autochthonous LAB against strains of *Bacillus* and *Pseudomonas* genera.

MATERIAL AND METHODS

The study included 7 LAB strains *Lactococcus lactis* ssp. *lactis* CNMN-LB-02, *Lactococcus lactis* ssp. *lactis* CNMN-LB-06, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-07, *Lactococcus lactis* ssp. *lactis* CNMN-LB-09, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14, *Streptococcus thermophilus* CNMN-LB-16, *Streptococcus thermophilus* CNMN-LB-17, stored in National Collection of Non-pathogenic Microorganisms (NCNM) at the Institute of Microbiology and Biotechnology of the Technical University of Rep. Moldova.

Rehydration of lyophilized LAB cultures after lyophilization was carried out in skim milk, following the standard method. This process involved rehydrating them with sterile skim milk and incubating them at a temperature of 30°C and 42°C for 3 hours to facilitate their revival. The viability of strains (expressed in CFU mL^{-1} colony forming units) was determined by the method of counting colonies.

Acidity was determined by titration with 0.1 N NaOH using phenolphthalein as an indicator, results in Thorner degrees (°T) were expressed. Coagulation activity was defined after incubation at 30 and 42°C within 4–6 h (fast), 6–12 h (medium), or >12 h (slow) time frames [1, 31].

After reactivation LAB strains are preserved through periodic subculturing using skim milk medium or hydrolyzed milk medium (HMM) following the standard method [6, 32]. Also as a laboratory medium HMM supplemented with 2% yeast extract, 2% sucrose and magnesium sulfate, potassium biphosphate in trace amounts (HMM + 2% Ys + 2% S) was used.

The antibacterial activities were tested on *Bacillus subtilis*, *Bacillus subtilis* var. *mesentericus*, *Bacillus cereus* var. *fluorescens*, *Pseudomonas fluorescens* test cultures, stored in NCNM, resulting from the fact that these bacteria are considered to be a reason for food contamination.Test cultures were subcultured by pour plate method. As a nutritional media for the cultivation and growth of mentioned bacterial strains, nutrient agar and King B medium were used. Cultivation was carried out at a temperature of 35±1°C for a period of 24-48 hours.

To assess antagonistic effects of selected LAB, the antibacterial activity was tested against *B. subtilis* CNMN-BB-01, *B.subtilis* var. *mesentericus* CNMN-BB-02, *B. subtilis* CNMN-BB-06, *B. cereus* var. *fluorescens* CNMN-BB-07, *B. subtilis* CNMN-BB-08, *B. subtilis* CNMN-BB-09, *B. subtilis* CNMN-BB-10, *P. fluorescens* CNM-PFB-01, *P. fluorescens* CNMN-PsB-02, *P.fluorescens* CNMN-PsB-11, *P. fluorescens* CNMN-PsB-12, and determined by well diffusion method on agar [12]. For well method, a quantity of 0,1 mL (bacterial suspension) obtained after cultivation of LAB in liquid HMM was placed into wells in Petri plates with medium already containing test bacteria. To allow a good diffusion of the LAB suspension into the agar medium, the plates were placed for 1 hour at room temperature before incubation. The diameter of the growth inhibition zones was measured after 24-48 hours of incubation [32].

RESULTS

To evaluate the viability of LAB stored in NCNM by the method of lyophilization, cultures were reactivated. The results of the microscopic examination, morphological, cultural and technological characteristics of reactivated strains were shown in previous studies [3, 5], the data describes selected 7 strains is presented below. It was found that all strains are viable, morphological and cultural changes did not occur and are according to the strain passport.

After rehydration selected strains were tested for parameters of acidifying activity and milk coagulation to confirm technological property, which are biotechnologically relevant for dairy industry. Consequently, fermentation time and acidity were determined. Strain *L. lactis* ssp. *lactis* CNMN-LB-06 produced higher levels of acid (119.3°T), followed by strain *L. lactis* ssp. *lactis* bv. *diacetylactis* CNMN-LB-07 (73.3°T). Despite having fast-medium coagulation activity (clotting time from 6–9.5 h), these strains generated high acidity levels (more than 70°T), indicating that these strains are active acid forming agents prospective not only for milk fermentation.

Obtained bacterial solutions were tested on test cultures. The antibacterial property of LAB stored in NCNM against *Bacillus* and *Pseudomonas* strains has not been studied before.

Determination of the antibacterial activity of LAB strains against *B. subtilis*, *B. subtilis* var. *mesentericus*, *B. cereus* var. *fluorescens*, *P. fluorescens* showed different sensitivity. Thus, the diameter of the inhibition zones against representatives of *P. fluorensens* varied within the limits of 13.0-25.7 mm,

Figure 1. Antibacterial activity of *L. lactis* ssp. *lactis* CNMN-LB-09 (9) strain against *B. cereus var. fluorescens* CNMN-BB-07 (07)

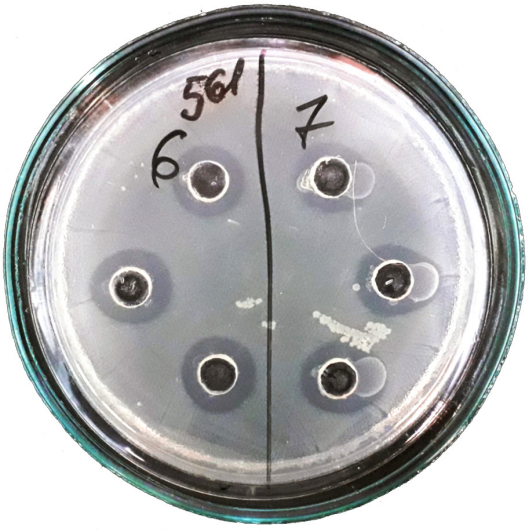

Figure 2. Antibacterial activity of *L. lactis* ssp. *lactis* CNMN-LB-06 (6) and *L. lactis* ssp. *lactis* bv. *diacetylactis* CNMN-LB-07 (7) strains against *P. fluorescens* CNMN-PsB-12 (561)

and against *Bacillus* inhibition zones varied from 10.7 mm to 16.7 mm. The studied strains *S.thermophilus* CNMN-LB-16 and *S. thermophilus* CNMN-LB-17 did not affect growth of tested strains (a nonsignificant sensitivity only against *B. cereus* var. *fluorescens* CNMN-BB-07 – 11.0 mm) (Table 1).

Mostly strains showed zones of 10.7-15.3 mm, considered an insignificant antibacterial activity (Fig. 1), with moderate inhibitory effects against *B.subtilis* CNMN-BB-06, *B. subtilis* CNMN-BB-09, *P. fluorescens* CNMN-PsB-02, *P. fluorescens* CNMN-PsB-11, *P. fluorescens* CNMN-PsB-12, and strong activity observed in *L. lactis* ssp. *lactis* bv. *diacetylactis* CNMN-LB-14 against *P. fluorescens* CNMN-PsB-02.

L. lactis ssp. *lactis* CNMN-LB-02 had no antibacterial activity to seven test cultures, but had the ability to retard the growth of *P. fluorescens* CNMN-PsB-02 with a zone up to 20.0 mm. The bacterial solution of the *L. lactis* ssp. *lactis* CNMN-LB-06 strain showed mostly insignificant sensitivity, the diameter of the inhibition zones varied from 12.3 mm to 14.7 mm, exhibited moderate inhibitory effect only against *P. fluorescens* CNMN-PsB-12 (zone of 16.0 mm) (Fig. 2).

L. lactis ssp. *lactis* bv. *diacetylactis* CNMN-LB-14 bacterial solution showed the ability to inhibit growth of majority test bacteria. It was also noticed in *L. lactis* ssp. *lactis* CNMN-LB-09 and *L. lactis* ssp. *lactis* bv. *diacetylactis* CNMN-LB-07 moderate inhibitory effects against *B. subtilis* CNMN-BB-06 (zone of 16.0 mm), *B. subtilis* CNMN-BB-09 (zone of 16.7 mm), *P. fluorescens* CNMN-PsB-02 (zone of 19.3 mm), *P. fluorescens* CNMN-PsB-12 (zone of 17.3 mm) (Fig. 2).

The most effective LAB strains were selected to improve the antibacterial property via cultivation in the HMM with adition of yeast extract and sucrose. In vitro evaluation of antibacterial activity was tested on *B. subtilis* CNMN-BB-06, *B. subtilis* CNMN-BB-09, *P.fluorescens* CNMN-PsB-02 and *P. fluorescens* CNMN-PsB-12 cultures most sensitive to the action of LAB bacterial suspension.

In order to establish the influence of addition of yeast extract and sucrose in the HMM, the viable cell count and antimicrobial activity of 3 strains *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-07, *L. lactis* ssp. *lactis* CNMN-LB-09, *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14 were studied and compared with their initial activity on the HMM. The results are shown in Table 2.

It was found that the metabolite solutions obtained during strains cultivation in the growth medium HMM supplemented with 2% of yeast extract and sucrose, showed the higher sensitivity against tested *Bacillus* and *Pseudomonas* compared to the initial one. Thus, the diameter of the inhibition zones varied from 12.3-20.0 mm (against *Bacillus*) to 15.7-27.0 mm (against *Pseudomonas*).

The highest antagonistic activity after cultivation in medium HMM + 2% Ys + 2% S was demonstrated by *L. lactis* ssp. *lactis* CNMN-LB-09 increased by 49% against *P. fluorescens* CNMN-PsB-02 and 17-20% against *B. subtilis*, respectively. Also the maximum inhibitory effect of the bacterial solution was demonstrated by *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14 after cultivation in enriched medium against *B. subtilis* CNMN-BB-09 (44%) and *P. fluorescens* CNMN-PsB-12 (26 %). The titer of CFU mL^{-1} after growing in a HMM medium supplemented with yeast extract and sucrose also increased from 10^8 to 10^9. Cultivation of the strain *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-07 in this medium registered an insignificant increase in the antibacterial activity and cell count, a sensitivity against test cultures constituted by 10-17% more than in HMM medium.

Table 1. Inhibition zone diameters of LAB strains against *Bacillus* spp. and *P. fluorescens*

Strain name	***L. lactis* ssp. *lactis* CNMN-LB-02**	***L. lactis* ssp. *lactis* CNMN-LB-06**	***L. lactis* ssp. *lactis* bv.*diacetylactis* CNMN-LB-07**	***L. lactis* ssp. *lactis* CNMN-LB-09**	***L. lactis* ssp. *lactis* bv.*diacetylactis* CNMN-LB-14**	***S. thermophilus* CNMN-LB-16**	***S.thermophilus* CNMN-LB-17**
B. subtilis CNMN-BB-01	0	14.7±1.3	12.7±0.7	14.3±1.3	13.7±1.3	0	0
B. subtilis var. *mesentericus* CNMN-BB-02	0	12.7±0.7	12.3±0.7	14.7±0.7	15.3±0.7	0	0
B. subtilis CNMN-BB-06	12.3±0.7	0	0	16.0±1.1	14.3±0.7	0	0
B. cereus var. *fluorescens* CNMN-BB-07	13.3±0.7	13.0±1.1	14.3±0.7	14.0±1.1	10.7±0.7	11.0±1.1	0
B. subtilis CNMN-BB-08	0	12.0±0.0	0	0	14.3±0.7	0	0
B. subtilis CNMN-BB-09	0	0	0	16.7±1.3	13.7±0.7	0	0
B. subtilis CNMN-BB-10	0	0	0	0	12.7±0.7	0	0
P. fluorescens CNM-PFB-01	0	0	14.7±0.7	13.7±0.7	0	0	0
P. fluorescens CNMN-PsB-02	20.3±0.7	13.0±0.0	19.3±3.5	12.3±0.7	25.7±1.7	0	0
P. fluorescens CNMN-PsB-11	0	11.7±0.7	12.7±0.7	13.0±1.1	16.3±0.7	0	0
P. fluorescens CNMN-PsB-12	14.7±2.4	16.0±1.1	17.3±1.3	14.0±1.1	15.3±0.7	0	0

Table 2. Antibacterial activity of LAB strains, cultivated in the hydrolized milk-based media, zone of inhibition

Strain name	Culture medium	Titer, $\log_{10}$ CFUmL^{-1}	***B. subtilis* CNMN-BB-06**		***B. subtilis* CNMN-BB-09**		***P. fluorescens* CNMN-PsB-02**		***P. fluorescens* CNMN-PsB-12**	
			mm	**% Control**	**mm**	**% Control**	**mm**	**% Control**	**mm**	**% Control**
L. lactis ssp. *lactis*	HMM	1.4± 0.2 x 10^8	0	-	0	-	19.3±3.5	100	17.3±1.3	100
bv.*diacetylactis* CNMN-LB-07	HMM+2%Ys+2%S	3.1± 0.3 x 10^8	12.3±0.7	-	17.7±1.7	-	21.3±2.4	110	20.3±1.3	117
L. lactis ssp. *lactis*	HMM	1.9± 0.2 x 10^8	16.0±1.1	100	16.7±1.3	100	12.3±0.7	100	14.0±1.1	100
CNMN-LB-09	HMM+2%Ys+2%S	5.2± 0.4 x 10^9	18.7±0.7	117	20.0±1.1	120	18.3±0.7	149	15.7±1.3	112
L. lactis ssp. *lactis*	HMM	2.0± 0.3 x 10^8	14.3±0.7	100	13.7±0.7	100	25.7±1.7	100	15.3±0.7	100
bv.*diacetylactis* CNMN-LB-14	HMM+2%Ys+2%S	5.7± 0.2 x 10^9	16.3±1.7	114	19.7±1.3	144	27.0±2.0	105	19.3±0.7	126

DISCUSSION

Lyophilization is the safest method for long-term conservation without risk of culture loss or changes in the initial morpho-cultural characteristics. Performing cell count immediately after lyophilization is a very important criterion, so high percentage of viable cells after lyophilization can ensure the viability of strains of biotechnological interest for a long time. According to the present study, viability of LAB strains after rehydration decreased relatively slowly, it was observed that the cell viability after lyophilization ranges between 87.1% to 94.7%, indicating a good index for cultures preserved for 15 years. The results obtained in our studies are consistent with the literature, which also noted that reactivated LAB strains stored for 3 to 24 years by vacuum-drying and from 27 to 54 years in a freeze-dried state have maintained their viability, culture characteristics and microscopic features [14].

Microorganisms of various systematic groups can differ significantly in their sensitivity to the same conditions, each strain requires an individual approach regarding media, cultivation and storage. Preservation and maintenance of strains viability (periodic subculturing) depends on cultivation medium used. Composition of culture medium has an important role for bacteria and can affect the growth of microorganism, their productivity and technological properties. Studied LAB strains had the ability to inhibit the growth of certain test bacteria due to synthesized inhibitory substances such as lactic acid and diacetyl. The highest results for *L. lactis* ssp. *lactis* CNMN-LB-09 and *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14 were obtained with HMM supplemented with sources of carbon and carbohydrates, against *P. fluorescens* CNMN-PsB-02 (Ø 18.3 mm – 49%), and *B. subtilis* CNMN-BB-09 (Ø 19.7 mm – 44%). According to literature analysis, the cultivation media based on hydrolyzed proteins, yeast

extract and carbohydrates are excellent nitrogen and nutrient sources for microbial culture, therefore growth medium supplemented with different nutrients can improve bacterial activity.

Industrial media based on peptone, beef and yeast extract such as MRS and M17 are the most commonly used standard media for LAB cultivation, but their cost and specific preparation steps can limit their uses for academic purposes (as a laboratory medium) and can be insufficient to promote the growth of all LAB and probiotics strains [11]. Authors Garg *et al.* [9] emphasized that MRS medium, being expensive for daily routine research, can be changed to low-cost Soyanutri Jaggery agar (SJA), used in laboratory conditions as effective alternative medium for LAB as shown by the higher number of colony growth. Thus, investigation the impact of nutrients on the growth of microorganism and optimization of culture media remain relevant today.

Investigations provided by Matevosyan *et al.* [18] showed that mixed cultures had a significant difference in antibacterial activity depending on the type of growth medium. Mixed LAB cultures cultivated in MRS medium demonstrated significant antibacterial activity only in the case of simultaneous cultivation of strains. However the inhibitory effect of many LAB associations was significantly increased in the case of separated cultivation in milk medium. The co-cultivation of some strains, cultivation media or duration of cultivation can lead to changes in antagonistic activity. This limitation aligns with our previous studies that suggest the need to research effects of different culture media or nutrients for enhancing the inhibitory effect of some LAB against phytopathogenic bacteria, such as *Agrobacterium tumefaciens*, *Corynebacterium michiganense*, *Xanthomonas campestris*, *Erwinia carotovora* in order to identify and substantiate new directions in the implementation of these microorganisms [2].

Results obtained by Raman [23] had shown that study of antimicrobial activity of LAB strains becomes more promising. The main mechanisms of LAB antimicrobial activity which can improve the quality and safety of food and help prevent it from spoilage are: the production of lactic, acetic, propionic, formic, succinic acids which reduce the pH; production of hydrogen peroxide, and production of bacteriocin [13]. While microbial contamination of food are commonly caused by biological agents such as *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, Marangoz *et al*. [16] and Moschonas *et al*. [20] discovered that *Bacillus* spp. can be responsible for spoilage of dairy products, as well as Marcelli *et al*. [17] showed that species of the genus *Pseudomonas* are the most common causative agents of meat spoilage.

Modern research are focused not only on food manufacturing process, but also on nutrient-rich animal feed. Organic acids produced by LAB are the important secondary metabolites that exhibit preservative effects not only in fermented food but in silage. Ensilage represent the most economically advantageous method of preserving feed - common way to preserve the moist forage crop, which could improve the feed quality and prolong storage dye to lactic acid fermentation. According to several studies, improving of biotechnology relevant strains and their using in additives will contribute to achieve positive effects in regulating ensilage process, especially for high-protein vegetable raw materials [10, 27]. High-quality feed is the basis of farm animal health and leads to increased weight gain in meat-producing animals, higher milk yields and improved reproductive performance in breeding stock [22]. The accumulation of spoilage microorganisms such as *Bacillus* and *Pseudomonas* in food and silage leads to animal, human disease as a result [21, 28, 29]. To prevent the contamination of such undesired microorganisms chemical preservatives are used, which can lead to health issues. Food industry suggest the need to develop alternative strategies - natural preservatives such as bioprotective LAB, to prevent microbial spoilage in natural products.

LAB strains stored in NCNM have technological important properties for industrial applications as starter cultures for dairy products. It is well known that LAB can synthesize antimicrobial substances with antimicrobial effect, but investigation of antibacterial activity of autochthonous LAB against *Bacillus* spp. and *P.fluorensens* strains have never been studied before.

Therefore, it can be concluded that autochthonous *L. lactis* ssp. *lactis* CNMN-LB-09 and *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14 possess a pronounced antibacterial activity against certain *P. fluorescens* and *B. subtilis*, give evidence for further investigation of LAB deposited in NCNM for their potential application to control food spoilage microorganisms.

In further studies to obtain positive results in antibacterial activity, need to pay close attention to the composition of the culture medium; the synthesis of substances with inhibitory effects such as bacteriocins, diacetyl, organic acids; as well as the study of other antimicrobial strains that can suppress better the growth of phytopathogens or pathogens.

Acknowledgements. This research was funded by Government of Republic of Moldova, Ministry of Education and Research, Research Subprogram 020101 “InBioS—Innovative biotechnological solutions for agriculture, medicine and environment”. Institutional financing contract no. 4/FI of 22 February 2024.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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Received: November 12, 2024
Accepted: April 24, 2025
Published Online: April 25, 2025
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House