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ANTIMYCOTIC ACTIVITY OF THE ISOLATES OF LACTOBACTERIA FROM WATER AND MUSSELS OF BLACK SEA

Aim. *Determination of the presence of antimycotic activity and its level in thirteen lactobacteria isolates from Black Sea water and mussels.* ***Methods.*** *Antimycotic activity of lactic acid bacteria (LAB) isolates was tested by agar diffusion method in 24-well plates. To determine character of action the plates were incubated for 7 days at 25 °C.* ***Results.*** *For the first time antimycotic activity of marine LAB from Odesa coast was estimated. The majority of the tested isolates of lactobateria exhibited antimycotic activity of high level completely inhibiting (in 100%) as mycelium growth as well as spore formation of Penicillium expansum UKM F-575 and Aspergillus niger UKM F-16706 on second day of the study. Four isolates (M.5.1, M.5.2, M.7.1, M.7.2) showed lower antimycotic activity (from 0 to 75%). Eight LAB isolates from seawater (W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4) and one isolate from mussel liquor (M4.1) completely inhibited mycelial growth and sporulation of P. expansum UKM F-575 and A. niger UKM F-16706 even within seven days indicating fungicidal character of action.* ***Conclusions.*** *The most of tested LAB isolates from Black Sea exhibited high antimycotic activity against P. expansum UKM F-575 and A. niger UKM F-16706 indicator strains. The most active lactobacteria isolates were W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4 isolated from Black Sea water and M4.1 – from mussel liquor. The character of the revealed antimycotic activity was determined as fungicidal.*

Key words: antimycotic activity, molds, mussels, lactic acid bacteria, Black Sea.

Lactic acid bacteria (LAB) are large group of microorganisms including representatives of various genera such as *Lactobacillus*, *Halolactobacillus*, *Marinilactobacillus*, *Pediococcus*, *Carnobacterium*, *Enterococcus* etc., which inhabit many ecological niches, including marine environment [1]. A great diversity of secondary metabolites including organic acids, bacteriocins, peptides and other compounds, which possess antimycotic activity are produced by lactobacteria [6].

It is known about great difference of marine environment compared to other ecological niches, primary by its extreme conditions indicating a possibility of isolation of bacterial strains with high antagonistic activity [2, 9]. Lactobacteria from marine environment can exhibit antimycotic activity and they can find possible application in food industry due to it [6].

Nowadays, consumers demand minimally preserved food combining with long storage time that can be dangerous for their health because of safety problems. Lactobacteria because of their antagonistic activity can be used as bioprotective microorganisms to preserve food products [5].

However, there is a limited amount of information regarding LAB from the Odesa littoral of Black Sea and their antimycotic properties, which indicates that they are understudied.

The aim of this work was to determine the presence of antimycotic activity and its level in thirteen lactobacteria isolates from Black Sea water and mussels.

Materials and methods

Eight LAB isolates which were isolated from the Black Sea water (W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4) and five ones isolated from *Mytilus galloprovincialis* Lamarck (M.4.1, M.5.1, M.5.2, M.7.1, M.7.2) were used in this study. Water and mussel samples were taken at the Hydrobiologycal station of ONU during the previous works. Classical MRS medium was used for the cultivation of LAB [4]. Lactobacteria were inoculated by the streak method and cultivated at 37 °C for two days. LAB strains were stored at +4 °C and maintained by periodic cultivation.

Before screening of LAB for the ability to produce antimycotic compounds, they were inoculated into MRS broth and cultured at 37 °C for 24 hrs. The concentration of cells of overnight cultures of lactobacteria was determined by the spectrophotometer SmartSpec Plus (Bio-Rad, USA) at the wavelength of 600 nm.

Also, two strains of molds were used for the study: *Aspergillus niger* UKM F-16706 and *Penicillium expansum* UKM F-575 from the collection of the D.K. Zabolotny Institute of Microbiology and Virology (Kyiv). Potato dextrose agar (PDA) was used for molds cultivation [10]. They were inoculated by the streak plate method in three sectors and grown at + 25 °C for three days. On the basis of three-day cultures of fungi grown on PDA, spore suspensions were prepared in sterile physiological solution, which were used as inoculation material [8].

After obtaining the suspensions, the number of spores in 1 mL was counted using a Horyaev camera and at a microscope magnification of 80X. The important stage was the standardization of the obtained concentrations of fungal spores in the suspensions, which was carried out by diluting the spore suspensions with a physiological solution to a final concentration of 10^4 spores/mL [8].

The diffusion into agar method was used to determine the antimycotic activity of marine LABs. For this, 1 mL of pre-melted 1.5% MRS agar was poured into the wells of 24-well plates. After solidification of the medium, 100 μL of the overnight cultures of lactobacteria with a known concentration of cells were added to each well. The culture of *Enterococcus italicus* ONU547 and MRS broth were used as the controls. Incubation was carried out for 48 hours at 37 °C. After that, 50 μL of molds spore suspension at a concentration of 10^4 spores/ml were added to each well and incubated at 25 °C for two days [8].

The results were estimated on the 2 nd day of the study. The percentage of the well area covered with mycelia and/or spores was visually assessed and, accord-

ingly, the percentage of inhibition of mycelial growth and inhibition of sporulation caused by LAB was determined. In the case of full absence of any mold signs the inhibition was estimated as 100%, if 50%, 75%, 70% of well area were covered with fungal mycelium or spores the inhibition was 50%, 25%, and 30%, respectively [8].

To study the character of action of marine lactobacteria, the plates with LAB and molds spores were placed in a thermostat and incubated at 25 °C for another five days. The presence and intensity of fungal mycelium growth, as well as sporulation were evaluated as described above. These criteria were noted every day and, accordingly, the level of the antimycotic activity of lactobacteria was determined.

All experiments were performed twice. Statistical data processing and graphs buildings were carried out in the program Microsoft Office Excel.

Results and discussion

Antimycotic activity of LAB isolates of marine origin

At the beginning of the experiment, the concentrations of overnight cultures of the LAB were measured using a spectrophotometer with a wavelength of 600 nm. The results of the study are shown in Table.

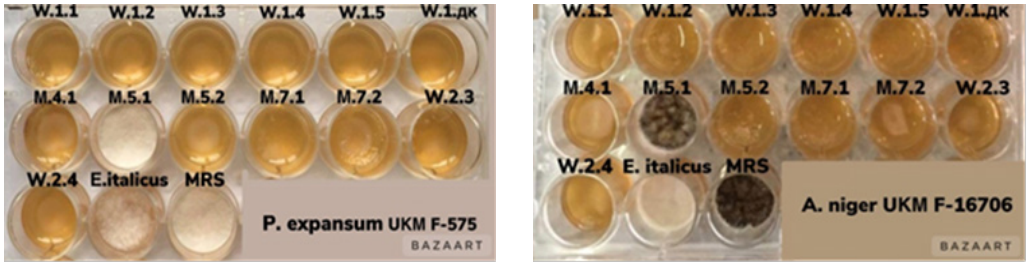
Table

Number of cells of overnight cultures of LAB of marine origin

LAB isolate	Number of cells, cells/ml
W.1.1	1.18×10^9
W.1.2	1.37×10^9
W.1.3	1.55×10^9
W.1.4	1.16×10^9
W.1.5	1.52×10^9
W.1.дк	1.20×10^9
M.4.1	1.44×10^9
M.5.1	5.61×10^8
M.5.2	1.69×10^9
M.7.1	1.57×10^9
M.7.2	1.95×10^9
W.2.3	1.61×10^9
W.2.4	2.18×10^9
MRS broth	0

It was estimated that the concentration of cells of all overnight cultures, except of M.5.1, was at approximately the same level.

In the study it was established that the most of the LAB isolates from marine sources showed high inhibitory activity against both *P. expansum* UKM F-575 and *A. niger* UKM F-16706 (Fig. 1).



a **b**

Fig. 1. Inhibitory activity of the isolates of marine lactobacteria against strains of molds *P. expansum* UKM F-575 (a) and *A. niger* UKM F-16706 (b) on second day of the study

Thus, the bacteria of isolates W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4 from the water of Black Sea and M.4.1 from the sea mussels completely inhibited the growth of the mycelium of the fungus *P. expansum* UKM F-575 on second day of the study (Fig. 2). Other isolates – M.5.2, M.7.1, and M.7.2 inhibited the growth of the molds in 75%, 70%, and 50%, respectively. These data are in agreement with the results of [3], where *Lactobacillus kefiri* M4 and *Pediococcus acidilactici* MRS-7 from dairy product (kefir) have demonstrated high antimycotic activity against *P. expansum* strains of fruit origin (apples and kiwi). Indeed, only one molds strain from the five tested – *P. expansum* LPH6 was resistant to the inhibitory substances of the studied lactobacteria strains. The scientists have also shown that organic and carboxylic acids of the studied LAB were mainly responsible for the antimycotic activity [3]. However, our publication is the first report on antimycotic effect of LAB from the Black Sea against molds *P. expansum*.

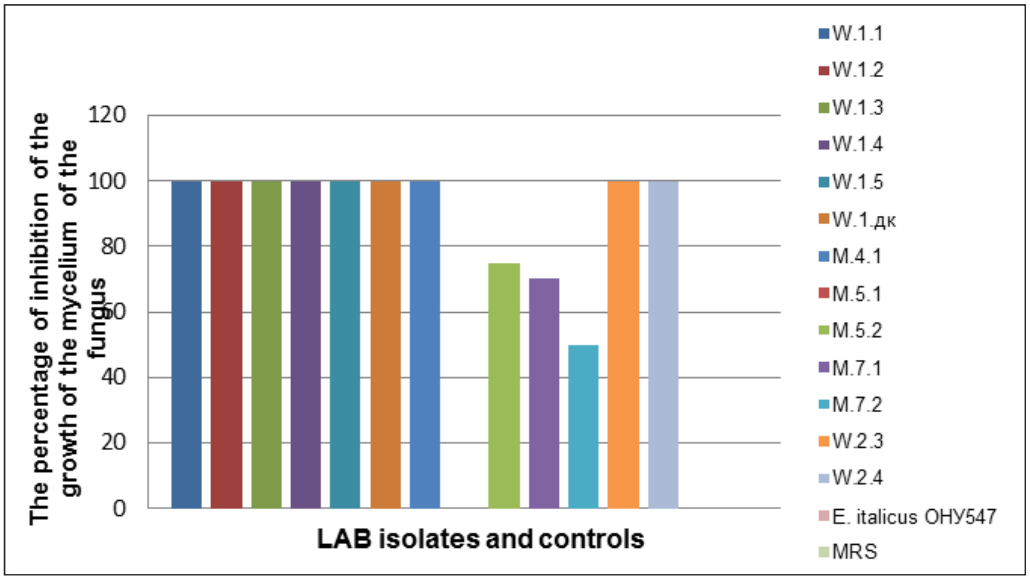


Fig. 2. Inhibitory effect of LAB from sea water and mussels on the growth of mycelium of *P. expansum* UKM F-575 on second day of the study

Antimycotic activity of lactobacteria isolates was also tested against a representative of molds of another species, namely *A. niger* UKM F-16706. This strain was more sensitive to the action of marine lactobacteria compared to *P. expansum* UKM F-575. Antimycotic activity of bacterial isolates of LAB W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4 from sea water and M.4.1, M.5.2, M.7.1, M.7.2 from sea mussels against *A. niger* UKM F-16706 was 100% on second day of the study (Fig. 3). However, this indicator strain was more resistant to the action of antimycotic compounds of isolate M.5.1 – the percentage of mycelial growth inhibition was only 50%. Our results are in agreement with data of Bulgarian scientists, who showed that LABs of the species *Lactobacillus plantarum*, which were also isolated from the Black Sea (*Mytilus galloprovincialis* Lam.), exhibited antimycotic activity against *A. niger*. Interesting, this mold species was the most sensitive to inhibitory compounds of marine lactobacteria among the tested *Penicillium claviforme*, *Candida albicans*, and *Saccharomyces cerevisiae* [6]. However, in other publication a low sensitivity of *A. niger* to LAB (of food origin) was reported [7].

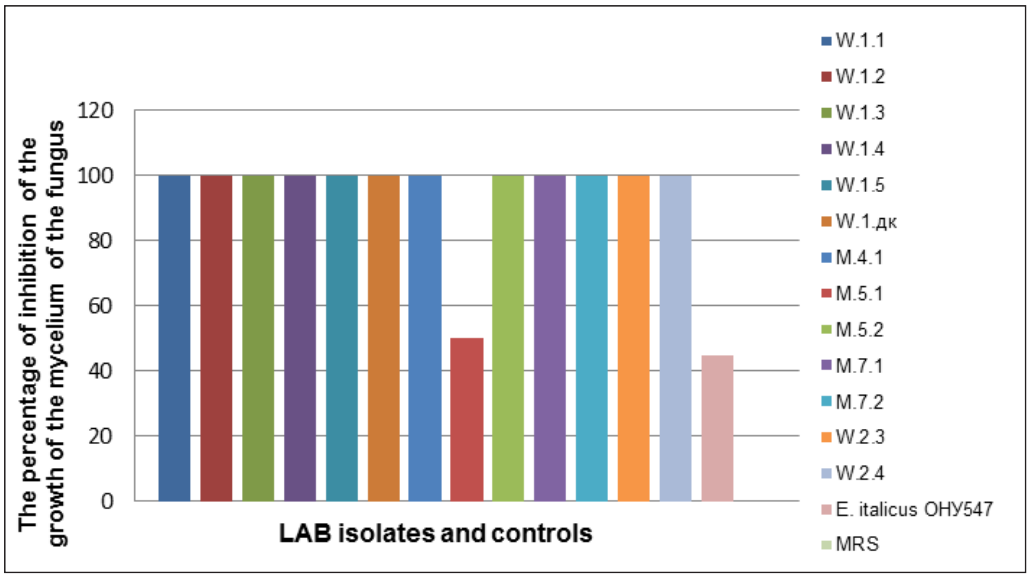


Fig. 3. Inhibitory activity of LAB isolates of marine origin against mycelial growth of *A. niger* UKM F-16706 on second day of the study

In addition to the mycelium growth inhibiting of the *A. niger* UKM F-16706, the studied strains of LAB also suppressed its sporulation (Fig. 4). All isolates, except of M.5.1, showed 100% inhibitory effect on sporulation.

Thus, as a result of our research, we found that isolates of lactobacilli both from the water of the Black Sea and from mussels showed high antimycotic activity and inhibited as mycelial growth, as well as sporulation of molds of two strains: *P. expansum* UKM F- 575 and *A. niger* UKM F-16706. All isolates of LAB from sea water are active producers of antimycotic compounds, which caused 100% inhibition of both *P. expansum* UKM F-575 and *A. niger* UKM F- 16706. LAB isolates from mussels showed a lower level of antimycotic activity.

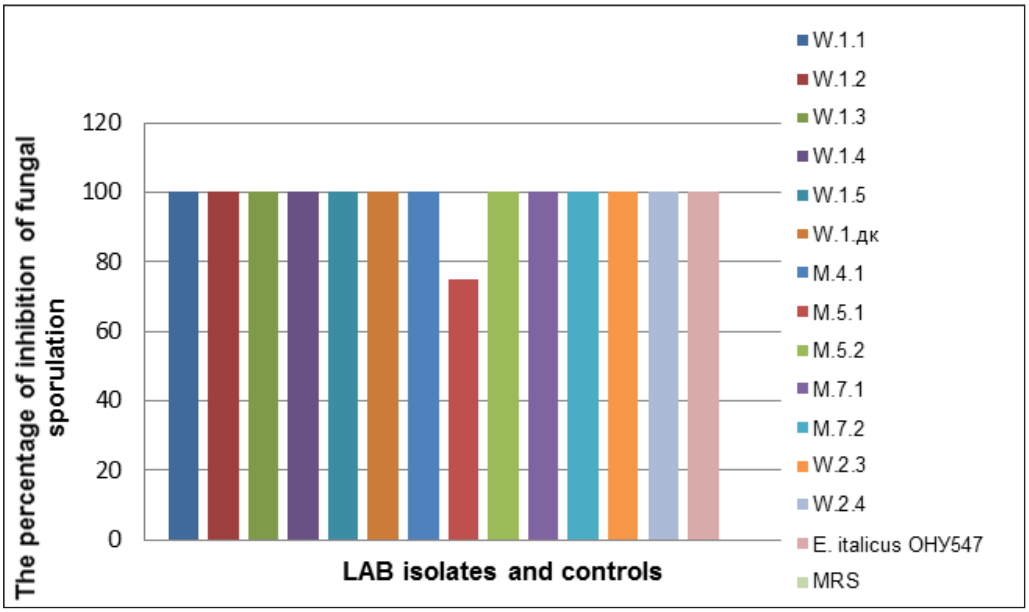
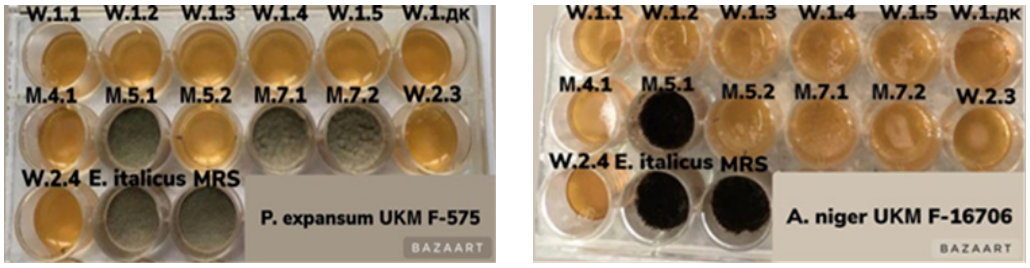


Fig. 4. Inhibition of sporulation of *A. niger* UKM F-16706 by strains of lactobacilli from sea water and mussels on second day of the study

Character of action of marine lactobacteria

The results of antimycotic activity on 3, 4, 5, 6, 7 days of fungi incubation at 25 °C were taken into account. It was established that a number of studied LABs retained their inhibitory activity even on the seventh day of incubation with the conditions optimal for fungi growth (Fig. 5). This may indicate the fungicidal character of the action of antimycotic compounds, which can be used in various areas of biotechnology.



a **b**

Fig. 5. Antimycotic activity of lactobacteria from seawater and mussels against molds *P. expansum* UKM F-575 (a) and *A. niger* UKM F-16706 (b) on the seventh day of the study

All LAB isolates from seawater and one (M.4.1) from mussels showed 100% antagonistic activity against the growth of *P. expansum* UKM F-575 mycelium, which lasted for seven days (Fig. 6). LAB isolates M.5.2, M.7.1, and M.7.2 on the third day of incubation inhibited the growth of the fungus in 50%, 50%, and 55%, respectively, but the inhibitory activity of strain M.5.1 during the same period was

0%. On the seventh day of incubation only the LAB M.5.2 among them retained antimycotic activity at the level of 50%.

In addition, the isolates W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, M.4.1, W.2.3, and W.2.4 completely suppressed the sporulation of *P. expansum* UKM F-575 for seven days (Fig. 7). Bacteria of isolate M.5.1, which was isolated from mussels, did not inhibit sporulation of this fungal strain.

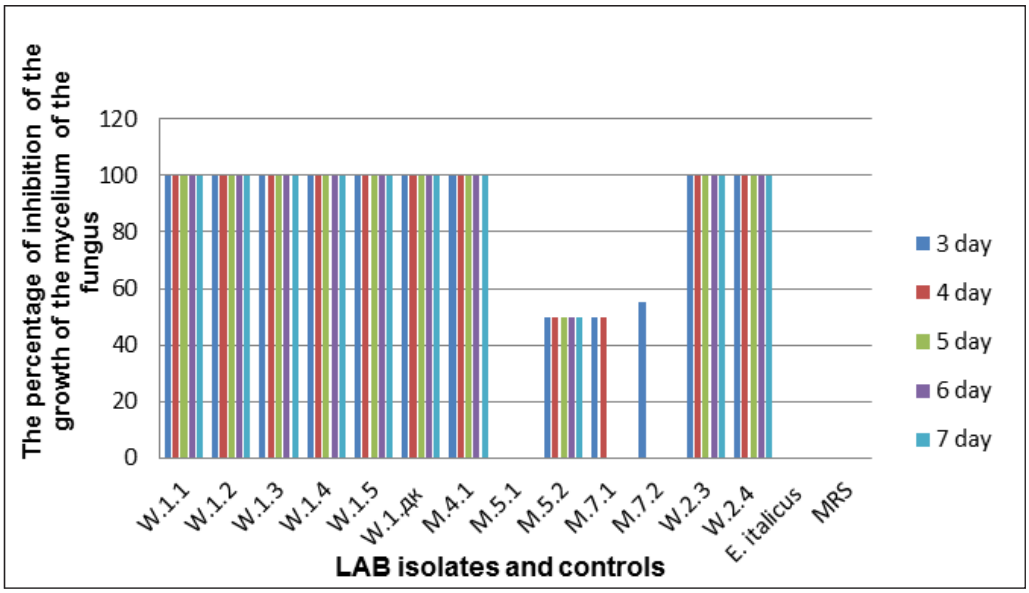


Fig. 6. Duration of antimycotic activity caused by LAB isolates against mycelial growth of *P. expansum* UKM F-575

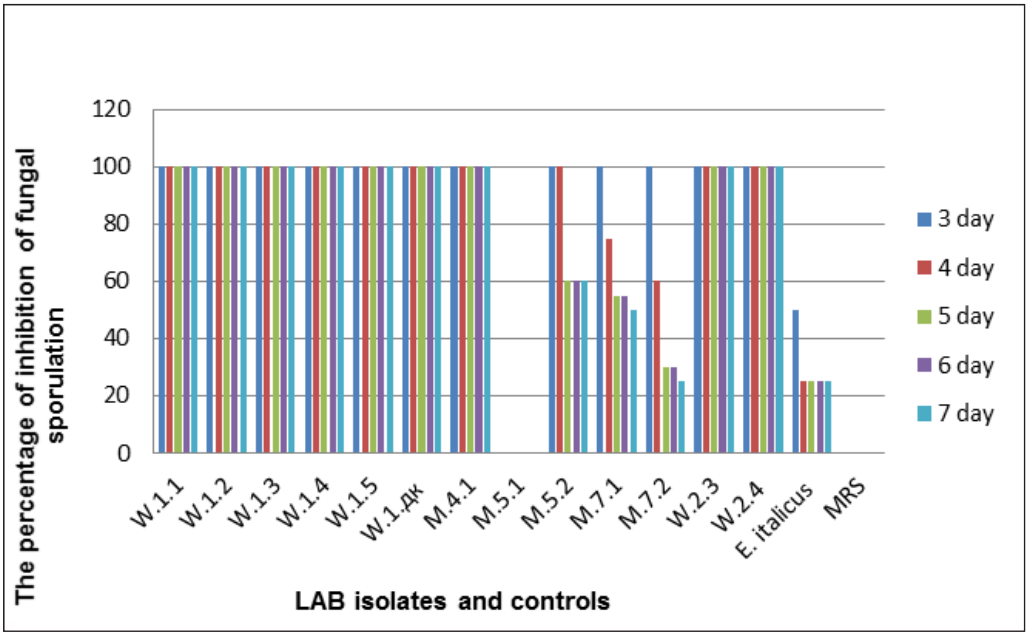


Fig. 7. Dynamics of inhibition of sporulation of *P. expansum* UKM F-575 by marine lactobacteria

In comparison with *P. expansum* UKM F-575, higher inhibitory activity of marine LABs was observed towards *A. niger* UKM F-16706, both in terms of mycelial growth and sporulation (Figs. 8, 9).

Thus, almost all the tested lactobacteria isolates completely inhibited the growth and sporulation of the fungus within seven days. Only bacteria of M.5.1 showed their antimycotic activity of lower level (50%).

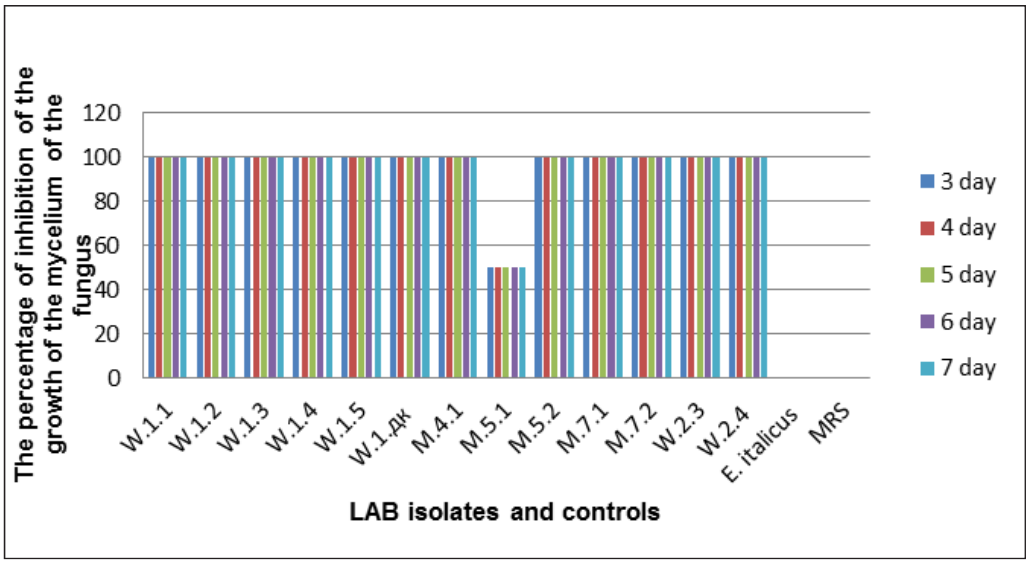


Fig. 8. Duration of inhibition of mycelial growth of *A. niger* UKM F-16706 caused by lactobacteria from sea water and mussels

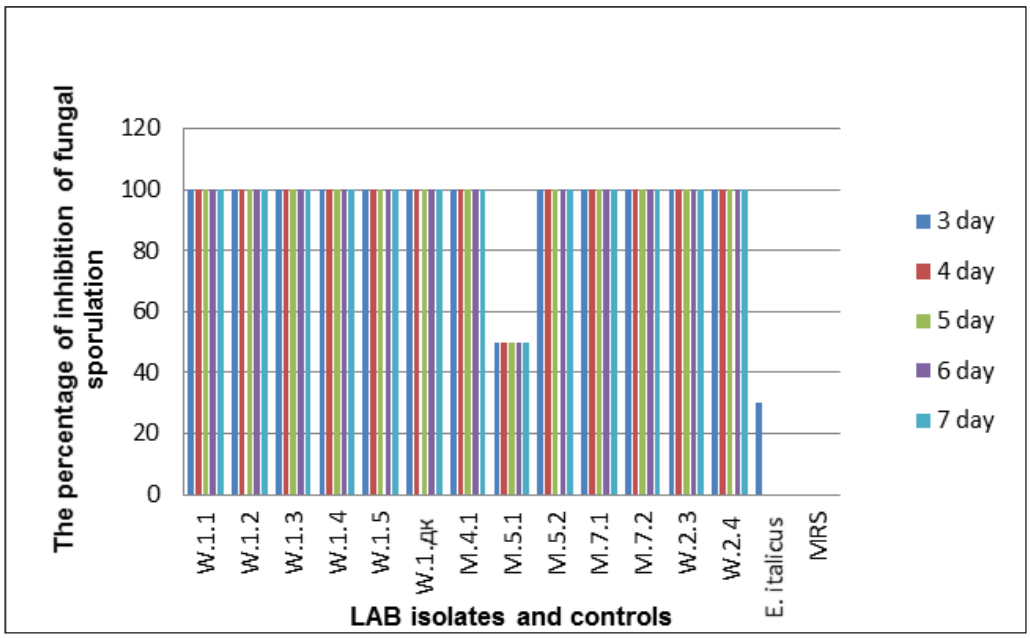


Fig. 9. Dynamics of inhibition of sporulation of *A. niger* UKM F-16706 under the effect of LAB from sea water and mussels

Therefore, our results indicate the presence of high number of LAB with antimycotic activity of fungicidal character of action in water of the Odesa Black Sea water area. Indeed, eight isolates of LAB from sea water (W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4) and one isolate from mussels (M.4.1) completely inhibited the growth and sporulation of the fungi *P. expansum* UKM F-575 and *A. niger* UKM F-16706 for seven days, thus exhibiting fungicidal effect. The new strains of lactobacteria of marine origin because of their high antimycotic activity can be perspective for use in biotechnology.

To our opinion, it is necessary to intensify the study of marine LABs not only from water and mussels, but also from other inhabitants of the Black Sea.

Conclusions

The majority of the isolates of lactobacteria from Black Sea have demonstrated antimycotic activity of high level against the tested indicator strains *P. expansum* UKM F-575 and *A. niger* UKM F-16706. The LAB isolates W.1.1, W.1.2, W.1.3, W.1.4, W.1.5, W.1.дк, W.2.3, W.2.4, and M4.1 were chosen as the most active. The studied LAB isolates from seawater of Black Sea showed fungicidal character of antimycotic activity against *P. expansum* UKM F-575 and *A. niger* UKM F-16706.

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АНТИМІКОТИЧНА АКТИВНІСТЬ ІЗОЛЯТІВ ЛАКТОБАКТЕРІЙ З ВОДИ ТА МІДІЙ ЧОРНОГО МОРЯ

Реферат

Мета. *Визначення наявності антимікотичної активності і ступеню її вираженості у тринадцяти ізолятах лактобактерій з чорноморської води та мідій.* ***Методи.*** *Антимікотичну активність ізолятів молочнокислих бактерій (МКБ) досліджували методом дифузії в агар в 24-лункових планшетах. Щоб визначити характер дії, планшети інкубували протягом 7 днів при 25 °С.* ***Результати.*** *Вперше оцінено антимікотичну активність морських МКБ з Одеського узбережжя. Більшість досліджених ізолятів лактобактерій виявили антимікотичну активність високого рівня, повністю пригнічуючи (на 100%) як ріст міцелію, так і спороутворення Penicillium expansum UKM F-575 та Aspergillus niger UKM F-16706 на другий день дослідження. Чотири ізоляти (М.5.1, М.5.2, М.7.1, М.7.2) показали меншу антимікотичну активність (від 0 до 75%). Вісім ізолятів МКБ з морської води (В.1.1, В.1.2, В.1.3, В.1.4, В.1.5, В.1.дк, В.2.3, В.2.4) та один ізолят із ліквору мідій (М4.1) повністю пригнічував ріст міцелію та спороутворення P. expansum УКМ F-575 та A. niger УКМ F-16706 навіть протягом семи днів, що свідчить про фунгіцидну дію.* ***Висновки.*** *Більшість досліджених ізолятів МКБ із Чор-*

ного моря проявили високу антимікотичну активність щодо індикаторних штамів P. expansum УКМ F-575 та A. niger УКМ F-16706. Найактивнішими ізолятами лактобактерій були В.1.1, В.1.2, В.1.3, В.1.4, В.1.5, В.1.дк, В.2.3, В.2.4 виділені з води Чорного моря та М4.1 – з ліквору мідій. Характер виявленої антимікотичної активності було визначено як фунгіцидний.

Ключові слова: антимікотична активність, цвілеві гриби, мідії, молочнокислі бактерії, Чорне море.

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