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Editorial office address:
D.K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine,
154 Academika Zabolotnoho Str., Kyiv, 03143, Ukraine
Tel.: +380 (44) 526 11 79
E-mail: microbiol.j@gmail.com
Web: http://www.microbiolj.org.ua

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RESEARCH ARTICLES

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**N.O. TYMOSHOK[1], O.A. DEMCHENKO[1], M.S. KHARCHUK[1],
V.S. BITYUTSKYY[2], O.S. TSEKHMISTRENKO[2], S.I. TSEKHMISTRENKO[2]**

[1] D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine
[2] Bila Tserkva national agrarian university,
8/1 Soborna squaire, Bila Tserkva, 09119, Ukraine
* Author for correspondence; e-mail: n_timoshok@ukr.net

STUDY OF GENUS *BACILLUS* (*B. CLAUSII*) PROBIOTIC BACTERIA REGARDING THE BIOGENIC EXTRACELLULAR SYNTHESIS OF SELENIUM NANOPARTICLES

The biogenic method of nanoparticle synthesis with the participation of microorganisms that are capable of producing nanomaterials of different shapes, sizes, and chemical compositions is a promising innovative direction of nanotechnology. Bacteria are chosen for the production of nanoparticles due to their rapid reproduction, ease of cultivation, low energy requirements, and minimal costs. The complex synthetic mechanisms available to microorganisms allow them to use a large number of building blocks to construct new biosynthetic nanostructures that can accumulate in vesicles inside the cell or by extracellular synthesis. In the modern world, the so-called «green» technologies come to the fore, and the active studies of microorganisms with a high enzymatic potential, which can be used in nanobiotechnology and are promising for practical application, are being actively expanded. We have screened strains of Bacillus bacteria for their ability to reduce Se (IV) in the composition of sodium selenite to Se^0. The ***aim*** *of the research was to study the processes of biogenic synthesis of selenium nanoparticles by probiotic strains of Bacillus clausii and their prospects for practical application.* ***Methods.*** *Cultivation of B. clausii was carried out in vials (500 cm^3) on a rotary shaker (20 rpm) at of 30 ^oC for 3 days on a nutrient medium of MPB. Sodium selenite 0.0065 g/100 mL was additionally added to the medium. A visual assessment of the color change in the nutrient culture medium was carried out under the conditions of its enrichment with 30 ppm Se in the composition of sodium selenite. The characteristics of nano-Se were studied using transmission electron microscopy (TEM).* ***Results.*** *It was established that the addition of sodium selenite 0.0065 g/100 mL (30 ppm Se) within the composition of sodium selenite to the nutrient medium revealed the ability of B. clausii to reduce oxyanions Se (IV) into nanoparticles of elemental selenium Se^0 (appearance of orange color). Bacterial cells and biosynthesized selenium*

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nanoparticles were separated for further transmission microscopy. Synthesized Nano-Se nanocrystals were detected in TEM images. Nano-Se particle sizes determined from TEM images varied within 298±52 nm. Nanoparticles obtained by B. clausii formed conglomerates of nanocrystals; individual nanoparticles had a spherical shape. A change in the color of the environment under the influence of Na_2SeO_3 during the cultivation of B. clausii was noted when the growth phase of the cultures went from logarithmic to stationary mode. Research has established for the first time that B. clausii is capable of reducing selenite to elemental selenium, as evidenced by TEM data. ***Conclusions****. The obtained data indicate the ability of B. clausii to reduce sodium selenite with the formation of extracellular selenium nanoparticles (Nano-Se). The B. clausii-assisted transformation of sodium selenite with extracellular deposits of Nano-Se opens an accessible source of biogenic Nano-Se and the creation of selenium-containing probiotic preparations based on it.*

Keywords*: biogenic synthesis, sodium selenite, selenium nanoparticles, probiotic strains, Bacillus clausii, TEM, extracellular synthesis.*

Statement of the problem and analysis of the recent research. Biogenic nanotechnology is an interdisciplinary field that combines biology and materials science. Green synthesis of nanoparticles using environmentally friendly methods and materials has become an important area of research in recent years. Nanoparticles are becoming more and more popular in various industries due to their unique properties and application possibilities. However, conventional methods of nanoparticle synthesis have several limitations, including the use of hazardous chemicals, high energy requirements, and high costs (Tao, Huachen et al., 2021). Green nanoparticles, are produced using environmentally friendly methods and materials and have several advantages over conventional nanoparticles. One of the main advantages of green nanoparticles is their biocompatibility. They are often made using non-toxic, biodegradable, and renewable materials, making them safe for use in a variety of applications. Green nanoparticles also have improved properties compared to conventional nanoparticles, such as increased stability and bioactivity. Another advantage of green nanoparticles is their cost-effectiveness. Traditional synthetic methods of nanoparticle synthesis can be expensive, but ecological synthesis methods are often more affordable. In addition, green nanoparticles are environmentally sustainable and contribute to sustainable development by reducing the use of hazardous chemicals and promoting the use of renewable resources. The biogenic method of nanoparticle synthesis is a particularly promising approach to obtaining green nanoparticles (Tymoshok et al., 2019). This method uses natural biological systems such as plants, animals, fungi, and bacteria to produce nanoparticles, resulting in a more environmentally friendly and cost-effective process. For example, plant extracts contain phytochemicals necessary for the synthesis of nanoparticles and for enhancing their bioactivity.

Nanoforms of selenium have attracted considerable attention due to their high bioavailability and lower toxicity compared to inorganic and organic forms of Se (Bokulich et al., 2016; Stewart et al., 2002; Zhang et al., 2008; The target program of fundamental research of the NASU 0120U102297, OK: 0222U004405). In addition, inorganic compounds of selenium are more toxic than organic ones (Gordon et al., 2010).

The development of an environmentally friendly and inexpensive method of synthesis of nanoparticles is of crucial importance. There are numerous organisms capable of synthesizing nanoparticles and having the potential to use them. Significant applications of nanomaterials are usually size-dependent, so size-controlled synthesis of nanomaterials is highly desirable.

Microorganisms are also increasingly being used to produce nanoparticles. Despite the unique reaction mechanism of each biogenic material, they all function essentially the same, resulting in the production of the desired nanostructures. In addition, biogenic synthesis can

be used to create biomimetic materials, which have advanced biomedical applications. The use of biopolymers, plant extracts, and biomolecules in green synthesis is of increasing interest. These reagents are biocompatible and function in a variety of ways, such as blocking, reducing, and modulating agents. Thus, the biogenic method of nanoparticle synthesis is a promising approach for obtaining green nanoparticles. The use of environmentally friendly procedures, such as the use of biopolymers, plant extracts, and biomolecules, is of increasing interest to achieve this goal.

Microorganisms can produce nanomaterials of different shapes, sizes, and chemical compositions. The complex synthetic mechanisms available to microorganisms allow them to use a large number of building blocks to construct new biosynthetic nanostructures that can accumulate in vesicles inside the cell or be transported outside. In this way, possible toxicological effects can be avoided. This inherently complex synthesis of nanomaterials has been a source of biological inspiration and biomimicry for many years. In particular, bacteria were chosen for the production of nanoparticles due to their rapid reproduction, ease of cultivation, low energy requirements, and minimal costs (Antezana et al., 2022). There are reports on the biosynthesis and most important properties of NPs (gold, silver, copper, selenium) obtained by bacterial methods, and the suggested intracellular and extracellular mechanisms of their bacterial biosynthesis are also discussed.

Molecular mechanisms of the production of selenium nanoparticles aerobically from selenite with the participation of the bacterium *Pseudomonas putida* KT2440 were presented for the first time in research (Avendaño et al., 2023). The obtained results indicate that the reduction of selenite to nanoselenium occurs through an interconnected metabolic network, which includes central metabolic reactions, in particular 2-oxoglutarate/glutamate metabolism (controlled by genes sucA, D2HGDH and PP_3148), sulfur metabolism (regulated by genes *cysG, sqr p do2, sqrR, ssuEF*,and *sfnCE*), as well as response to oxidative stress (*Gqr, lsfA, ahpCF,* and *sadI* genes). Importantly, these steps are combined to produce glutathione. Mutations affecting sulfite reductase activity reduced the bacteria's ability to transform selenite into nanoselenium. Scientists isolate genes that were not linked to selenium metabolism in other bacteria and focus on genes of biotechnological interest (sqrR, pdo2, and sqr), whose inactivation results in the production of selenium nanoparticles at a higher rate than the original strain bacteria. This provides prospects for the development of biotechnological tools to increase the efficiency of bionanotechnology for the production of nanoselenium with the participation of bacteria. Transcriptome studies have proven the effect of selenite on increasing the expression of membrane-associated proteins, and these changes in the expression patterns of membrane proteins are explained by the fact that the restoration of selenite leads to the accumulation of extracellular selenium nanoparticles and causes the rearrangement of the membrane proteome.

Recently, the first attempts were made to synthesize selenium nanoparticles, which were obtained extracellularly using the probiotic bacteria *B. clausii*, and their antibacterial effectiveness against multiresistant bacteria was evaluated. Biosynthesized Nano-Se showed antibacterial activity against most multidrug-resistant (MDR) bacteria, such as *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumonia, Acinetobacter baumannii, Proteus mirabilis, Pseudomonas aeruginosa*, and *Shigella sonii* in comparison with the results of the antibiotic study of ceftriaxone, azithromycin, cefepime, amikacin, meropenem, oxacillin, gentamicin, and ciprofloxacin. The authors envisage the use of probiotics and Nano-Se to enhance or replace antibiotics (Al-Shemmary et al., 2022). Wide enzymatic activity of *B. clausii* and the presence of intracellular sulfite reductase and nitrate reductase enzymes were established (Beller et al.,

2013). The presence of NADPH-dependent enzymes, including nitrate reductase, which plays a key role in the formation of nanoparticles, was revealed in the structure of *B. clausii*. The ability of *B. clausii* bacteria cultured from probiotic Enterogermin (Sanofi India Ltd.) to the extracellular biosynthesis of silver nanoparticles due to the significant nitrate reductase activity has been investigated by (Nadaf et al., 2019).

Currently, *Bacillus* cultures *coagulans*, *subtilis*, *clausii*, *toyoi*, *lichemiformis*, *mesentericus*, and *polymyxa* are included in probiotic preparations.

Enzymes produced by *B. subtilis* and *B. licheniformis* contribute to the improvement of digestion and assimilation of food, as well as the cleansing of wounds and inflammatory foci from necrotized tissues. The ability of *B. subtilis* IMB B-7393 and IMB B-7392 strains to reduce Se (IV) in the form of sodium selenite to Se^0 (Vaidyanathan et al., 2010; Tymoshok et al., 2019) and to accumulate more than 1 mM of sodium selenite was revealed. Such reports served as a basis for further research on the ability of *B. clausii* to transform sodium selenite into an elemental Se state.

It was shown that for the culture of *B. clausii*, NADPH-dependent nitrate reductase is responsible for the biosynthesis of nanosilver. There is a report on the ability of *B. clausii* to biosynthesize gold nanoparticles (Beller et al., 2013; Shi et al., 2011).

By changing the color of the medium, the cultivation of *B. clausii* in the presence of sodium selenite 0.006 g/100 mL, i.e. 30 ppm Se, led to the appearance of an orange-brick color, which indicated the reduction of Se (IV) and the formation of Nano-Se.

All these studies have become the basis for studying the enzymatic potential of *B. clausii* for the synthesis of Nano-Se, since previously the culture of *B. clausii* was not used specifically for the biosynthesis of Nano-Se.

The **purpose** of the research was to study the processes of biogenic synthesis of selenium nanoparticles by probiotic strains of *B. clausii*.

Materials and Methods. Cultivation of *B. clausii* was carried out in vials (500 cm^3) on a rotary shaker (20 rpm) at a 30 ºC for 3 days of cultivation on the MPB nutrient medium. Sodium selenite was additionally added to the medium in a dose of 0.0065 g/100 mL. A visual assessment of the color change in the nutrient culture medium was carried out under the conditions of its enrichment with 30 ppm Se in the composition of sodium selenite. Nano-Se characteristics were studied by transmission electron microscopy (TEM) using a JEM-1400 electron microscope (Japan).

Results. This research has established for the first time that *B. clausii* is capable of reducing selenite to elemental selenium, which is evidenced by the appearance of orange color in the medium (Fig. 1).

The addition of sodium selenite in the amount of 0.0065 g/100 mL (30 ppm Se) within the composition of sodium selenite to the nutrient medium revealed the ability of *B. clausii* to reduce Se (IV) oxyanions and the formation of an orange color. A change in the color of the medium under the influence of Na_2SeO_3 during the cultivation of *B. clausii* was noted in the case of transition from the logarithmic phase of culture growth to the stationary one, which is consistent with the literature data (Wang et al., 2007). Further research on Nano-Se was carried out after the purification of the nanoforms by centrifugation.

To confirm the Nano-Se formation and clarify the morphology of nanoparticles, TEM analysis was performed. For the first time, based on the analysis of data on the involvement of the nitrate reductase enzyme in the synthesis of nanoparticles, we confirmed the formation of Nano-Se during the transformation of sodium selenite by the *B. clausii* culture (Fig. 2).

TEM was conducted to find out the microstructural features of the obtained samples.

TEM image analysis showed that the synthesized electron-dense Nano-Se particles existed extracellularly in the microenvironment of cells of the *B. clausii* strain in the form of nanoag-

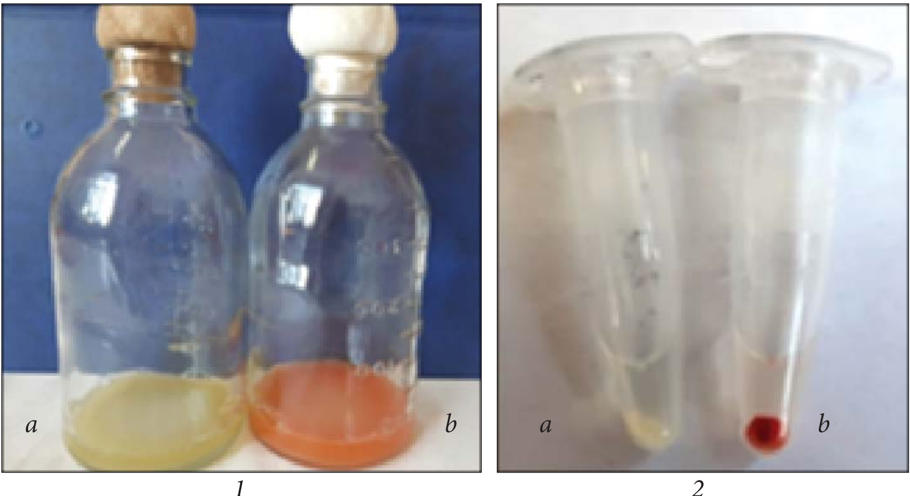

Fig. 1. The formation of nanoselenium particles under the conditions of 72-hour cultivation of *B. clausii* in a medium containing 30 ppm Se in the composition of sodium selenite (*1*) and purification of the obtained nanoforms by centrifugation (*2*): *a* — control; *b* — experiment

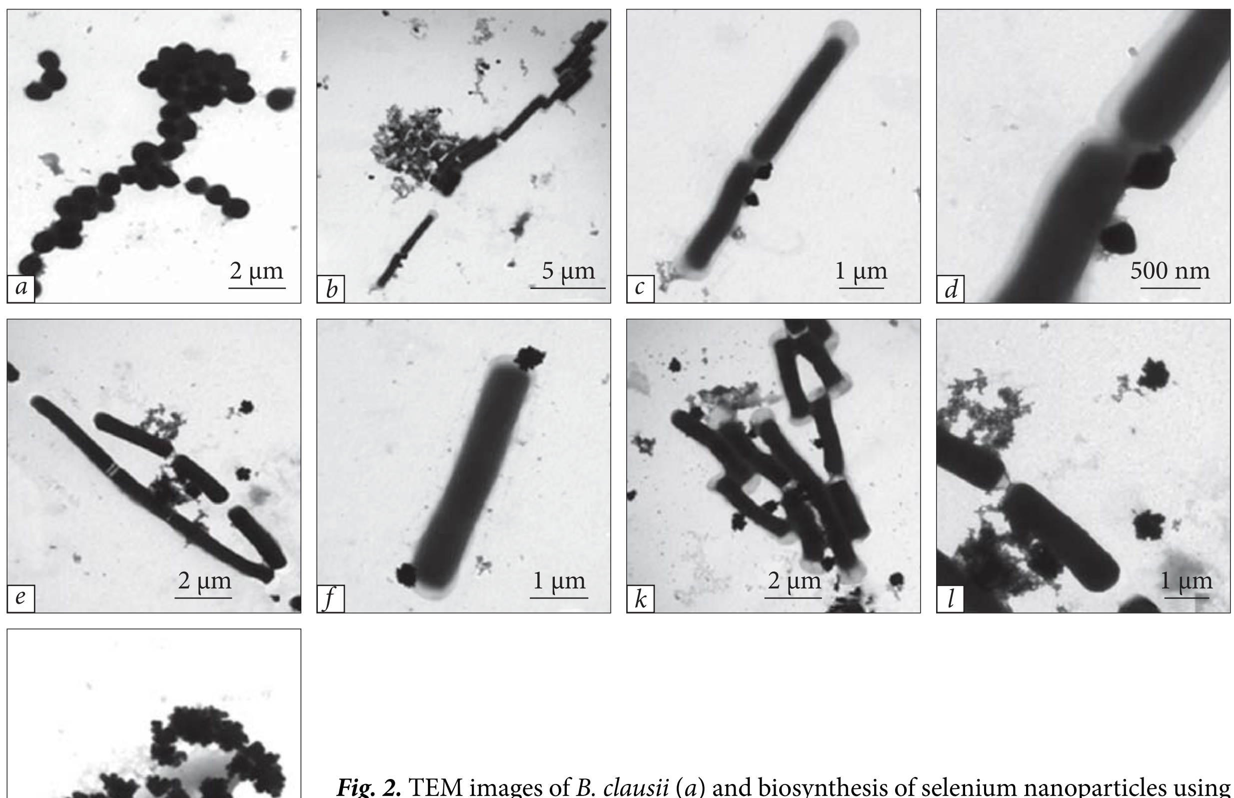

Fig. 2. TEM images of *B. clausii* (*a*) and biosynthesis of selenium nanoparticles using *B. clausii* (*b—m*) in the case of Nano-Se extracellular formation by the strain *B. clausii* under the conditions of enrichment of the culture medium with 30 ppm Se in the composition of sodium selenite. TEM revealed the presence of electron-dense Nano-Se particles and the formation of nanoagglomerates localized extracellularly (*b—m*)

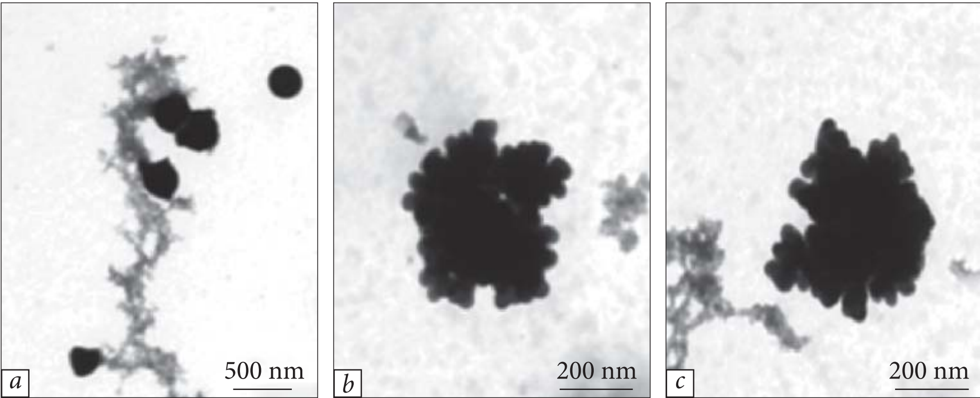

Fig. 3. TEM images of nanoparticles produced by *B. clausii* under the conditions of enrichment of the culture medium with 30 ppm Se in the composition of Na_2SeO_3

gregates. That is, when enriching the culture medium with 30 ppm Se (IV) and cultivating aerobically for 48 h at 30 °C, these extracellular electron-dense NPs are agglomerated.

The formation of sediment by *B. clausii* culture is shown for the first time. The transformation of *B. clausii* sodium selenite with extracellular deposits of nanoselenium opens an accessible source of biogenic Nano-Se and the creation of selenium-containing probiotic preparations based on it.

A separate part of the work was devoted to obtaining Nano-Se, i.e. separation of bacterial cells and formed nanoparticles for further transmission microscopy. Synthesized conglomerates of Nano-Se nanocrystals can be distinguished in TEM images (Fig. 3). Nano-Se particle sizes determined from TEM images vary within 298±52 nm.

Nanoparticles formed by *B. clausii* form conglomerates of nanocrystals. Some nanoparticles have a spherical shape. A change in the medium color under the influence of Na_2SeO_3 during the cultivation of *B. clausii* was noted during the transition from the logarithmic phase of culture growth to the stationary phase.

The reduction of Se in SeO_4^{2-} to SeO_3^{2-} is mainly catalyzed by soluble or membrane-bound selenate reductase (Ser), which combines three subunits that have molybdenum as a cofactor located in the periplasm or on the cytoplasmic membrane.

Discussion. Due to the presence of NADPH-dependent nitrate reductase enzymes, which are involved in the biosynthesis of nanoparticles, *B. clausii* is able to form silver and gold NPs. NADH-dependent nitrate reductase, which acts as an electron shuttle, accepts electrons from nitrate and transfers them to the metal ion to form nanoparticles, as illustrated for *F. oxysporum*, *P. aeruginosa*, and other microorganisms (Kalimuthu et al., 2008; Mikhailova et al., 2020; Mohd Yusof et al., 2019).

Involvement of transmembrane respiratory nitrate reductase (Nar) (Downey, 1966) and periplasmic nitrate reductase (Nap) (Tao, Huachen et al., 2021) are very important in the biosynthesis of NPs (Gupta, 1997). It should be noted that the active center of these enzymes is molybdenum. In particular, a membrane-bound nitrate reductase was found in the bacterium *Bacillus licheniformis*, which is involved in the synthesis of silver nanocrystals (Kumar et al., 2007). At the same time, the recovery of metals or metalloids occurs in response to the introduction of the corresponding ions and is caused by the stress reaction of the microorganism to a change in the environment (Mukherjee et al., 2018).

The research results show that *B. clausii*, which was cultivated from the probiotic *Enterogermin* (Sanofi India Ltd.), is appropriate to use for obtaining probiotic selenium-containing preparations, since *B. clausii* is capable of transforming sodium selenite into an elemental state.

In the modern world, the so-called «green» technologies take first place, and the active studies of microorganisms that have a high enzymatic potential to be used in nanobiotechnology and are promising for further research and practical application are being conducted. We screened strains of *Bacillus* bacteria for their ability to reduce Se (IV) in the form of sodium selenite to Se^0, which required additional research, including by TEM.

Our research showed for the first time the ability of *B. clausii* to reduce sodium selenite with the formation of Nano-Se. However, the confirmation of the probability of involvement of the selenate reductase enzyme of the culture of this strain in the recovery of Se (IV) remains unclear. For *Thauera selenatis*, the ability of periplasmic nitrate reductase to catalyze the reduction of selenite to elemental selenium was shown. In addition, the ability of *B. clausii* to form silver nanoparticles with the help of nitrate reductase was proven. In addition, anaerobic respiration of *Bacillus stearothermophilus* has been shown even earlier due to inducible nitrate reductase (Fajt et al., 2009).

In the applied aspect, it should be noted that the enzymes nitrate reductase (NaR) and nitrite reductase (NiR) are able to ensure the extracellular synthesis of nanoparticles (Bharti et al., 2020). Nitrate reductase converts nitrate to nitrite. The enzyme complex of the denitrifying bacteria *T. pantotropha* has two types of nitrate reductase enzymes, i.e., the membrane-bound NaR enzyme, which is active only under anaerobic conditions, the periplasmic NaR enzyme (active under aerobic conditions), and the nitrite reductase (NiR) enzyme, which reduces nitrite to N_2 (Hallol et al., 2013).

Some authors suggest using *Bacillus licheniformis* culture and its enzymatic potential, namely nitrate reductase, for the reduction of Ag^+ and the production of silver nanoparticles. In particular, the widespread mechanism of silver biosynthesis with the participation of bacteria is the action of the enzyme nitrate reductase. For *Bacillus licheniformis*, during bacterial reduction, alpha-nicotinamide-adenine-dinucleotide phosphate reduced form of NADPH-dependent nitrate reductase is involved. In the case of reduction, the nitrate turns into nitrite, and the electron is transferred to the silver ion; therefore, the silver ion (Ag^+) is reduced to Ag^0 (Jeevan et al., 2012; Vogel et al., 2018).

Conclusions. The obtained data indicate the ability of *B. clausii* to reduce sodium selenite with the formation of extracellular selenium nanoparticles (Nano-Se). For the *B. clausii* culture, the involvement of a nitrate reductase in the biosynthesis of silver nanoparticles has been proven; in our research, the ability of *Bacillus clausii* to transform selenite into extracellular Nano-Se was established for the first time (the target program of fundamental research NASU 0120U102297, OK: 0222U004405). However, further analysis and determination of the profile of the reductase enzyme and the understanding of the enzyme activity at the proteomic level will help to develop more effective strategies and practices for obtaining nanoparticles of extracellular localization. Therefore, the following experiments are aimed at comparative studies of the ability to transform sodium selenite and the formation of extracellular Nano-Se by different probiotic strains of the genus *Bacillus.*

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Conflicts of interest. The authors declare no potential conflict of interest concerning the article.

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Н.О. Тимошок[1], О.А. Демченко[1], М.С. Харчук[1],
В.С. Бітюцький[2], О.С. Цехмістренко[2], С.І. Цехмістренко[2]

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[2] Білоцерківський національний аграрний університет,
площа Соборна, 8/1, Біла Церква, 09119, Україна

ДОСЛІДЖЕННЯ ПРОБІОТИЧНИХ БАКТЕРІЙ РОДУ *BACILLUS (B. CLAUSII)* ЩОДО БІОГЕННОГО ЕКСТРАЦЕЛЮЛЯРНОГО СИНТЕЗУ НАНОЧАСТИНОК СЕЛЕНУ

Біогенний метод синтезу наночастинок за участі мікроорганізмів, які мають здатність виробляти наноматеріали різної форми, розміру та хімічного складу, є перспективним інноваційним напрямком нанотехнологій. Бактерії обираються для виробництва наночастинок завдяки їх швидкому розмноженню, простоті вирощування, низьким енергетичним потребам і мінімальним витратам. Складні синтетичні механізми, доступні мікроорганізмам, дозволяють їм використовувати велику кількість будівельних блоків для побудови нових біосинтетичних наноструктур, які можуть накопичуватися у везикулах всередині клітини, або шляхом екстрацелюлярного синтезу. У сучасному світі на перше місце виходять так звані «зелені» технології, проводиться активне вивчення та використання мікроорганізмів, що мають високий ферментативний потенціал, можуть використовуватися в нанобіотехнології та є перспективними для практичного застосування. Нами було проведено скринінг штамів бактерій роду *Bacillus* за здатністю до редукції Se (IV) у складі селеніту натрію до Se^0. **Мета.** Дослідити процеси біогенного синтезу наночастинок селену пробіотичними штамами *Bacillus clausii* та їх перспективи щодо практичного застосування. **Методи.** Культивування *B. clausii* проводили у флаконах (500 $см^3$) на ротаційному шейкері (20 об/хв.) за температури 30 °C впродовж 3-х діб на поживному середовищі МПБ. У середовище додатково вносили селеніт натрію 0.0065 г/100 мл. Проводили візуальну оцінку зміни кольору поживного середовища культури за умов його збагачення 30 ppm Se у складі селеніту натрію. Характеристики Nano-Se вивчали за допомогою трансмісійної електронної мікроскопії (ТЕМ). **Результати.** Внесення селеніту натрію 0.0065 г/100 мл (30 ppm Se) до поживного середовища виявило здатність *B. clausii* до редукції оксианіонів Se (IV) у наночастинки елементарного селену (Se^0) та утворення помаранчевого забарвлення. Проводили сепарацію бактеріальних клітин та біосинтезованих наночастинок селену для подальшого ТЕМ аналізу. На ТЕМ-зображеннях виявлено синтезовані нанокристали Nano-Se. Розміри частинок Nano-Se, визначені з ТЕМ зображень, варіюють у межах 298 ± 52 нм. Наночастинки, утворені *B. clausii*, формують конгломерати нанокристалів; окремі наночастинки мають сферичну форму. Зміну кольору середовища під впливом Na_2SeO_3 при культивуванні *B. clausii* відмічали при переході від логарифмічної фази зростання культур до стаціонарної. Дослідженнями вперше встановлено, що *B. clausii* здатні до редукції селеніту до елементарного селену, про що свідчать дані ТЕМ. **Висновки.** Отримані дані свідчать про здатність *B. clausii* до редукції селеніту натрію з утворенням позаклітинних наночастинок селену (Nano-Se). Трансформація селеніту натрію під впливом *B. clausii* відкриває доступне джерело біогенного Nano-Se для створення селеновмісних пробіотичних препаратів на його основі.

Ключові слова: *біогенний синтез, селеніт натрію, наночастинки селену, пробіотичні штами, Bacillus clausii, ТЕМ, позаклітинний синтез.*

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A.Z. PYLYPETS[1], I.I. KOVALCHUK[1,2*],
L.P. BABENKO[3], R.S. FEDORUK[1], T.M. KHYMYNETS[2], M.M. TSAP[1],
M.M. ROMANOVYCH[1], R.L. ANDROSHULIK[1], T.V. ZHYLIKHOVSKA[3]

[1] Institute of Animal Biology, NAS of Ukraine,
38 V. Stus Str., Lviv, 79034, Ukraine
[2] Stepan Gzhytsky National University of Veterinary Medicine and Biotechnologies,
50 Pekarska Str., Lviv, 79010, Ukraine
[3] D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine
* Author for correspondence; e-mail: irenakovalchuk@ukr.net

EFFECT OF DIFFERENT DOSES OF GE CITRATE AND PROBIOTIC *LACTOBACILLUS CASEI* B-7280 ON THE PHOSPHOLIPID COMPOSITION OF BEES TISSUES

Aim. *To investigate changes in the phospholipid composition of bee body tissues under the influence of the lyophilized probiotic strain Lactobacillus casei IMV B-7280 in combination with nanotechnological Ge citrate in laboratory conditions.* ***Methods.*** *The research was conducted on honey bees of the Carpathian breed. Bees of the control group were fed with 60% sugar syrup in the amount of 1 cm^3/group/day. Experimental 1 group of bees (E 1), in addition to 1 cm^3 of sugar syrup, received 0.1 μg of Ge in the form of nanotechnological citrate and a solution of the probiotic L. casei B-7280 at a concentration of 10^6 CFU/cm^3; experimental group 2 of bees (E 2), in addition to 1 cm^3 of sugar syrup, received 0.2 μg of Ge in the form of citrate and L. casei B-7280 at a concentration of 10^6 CFU/cm^3. Drinking sugar syrup, Ge citrate, and probiotics lasted 34 days. In the preparatory period and at the end of the experimental period, live bees were selected from the control and experimental groups for physiological and biochemical studies to determine the content of total phospholipids and the ratios of their classes in tissue homogenates of the entire organism. The content of total phospholipids was determined by the amount of inorganic phosphorus in the lipid extract. Thin-layer chromatography on silica gel was used to separate phospholipids. Rf values identified individual phospholipids. Quantitative analysis of phospholipid subclasses was performed using the TotalLab software, which was expressed as a percentage of total content.* ***Results.*** *The research results showed that in the homogenates of bee body tissues in the research groups, an increase*

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in the content of total phospholipids was established relative to the preparatory period. In the fractional composition of phospholipids, an increase in the content of phosphatidylethanolamine and phosphatidylcholine was established in the tissues of bees of groups E 1 and E 2 concerning the preparatory period. An increase in the content of phosphatidylethanolamine and phosphatidylcholine and a decrease in phosphatidylinositol in body tissue lipids of group E 2 compared to the control group were noted. A decrease in the content of phosphatidylinositol in the tissues of group E 2 bees relative to the preparatory period was also established. The content of sphingomyelin and lysophosphatidylcholine decreased in tissue lipids of bees of groups E 1 and E 2 as compared to the preparatory period. The use of lactobacillus casei strain B-7280 and Ge citrate led to an increase in the number of lactobacilli and bifidobacteria in both parts of the intestine, as well as to a decrease in the number of staphylococci, streptococci, and microscopic fungi. ***Conclusions.*** *Nanotechnological Ge citrate and probiotic L.casei in the applied doses under the conditions of their feeding with sugar syrup in a laboratory thermostat for 34 days show a dose-dependent biological effect on honey bees by increasing the content of total phospholipids and changing the ratios of individual subclasses of phospholipids. However, it also indicates a shift in the spectrum of different fractions of phospholipids to a decrease in the content of hard-to-oxidize lysophosphatidylcholine and sphingomyelin while increasing easy-to-oxidize phosphatidylcholine and phosphatidylethanolamine, which may indicate stabilization of compensatory mechanisms for supporting cell membranes. The use of Lactobacillus casei B-7280 and Ge citrate for feeding bees under the conditions of the laboratory thermostat led to quantitative changes in the composition of the intestinal microbiota of bees, in particular to an increase in the number of lactic acid bacteria and bifidobacteria, as well as a decrease in the number of some other groups of microorganisms in the intestine.*

Keywords: *honey bees, body tissues, nanotechnological Ge citrate, probiotic, phospholipids.*

The honey bee (*Apis mellifera*) is an essential agricultural pollinator of entomophilous crops, increasing their yield. However, factors of biotic, abiotic, and anthropogenic origin disrupt physiological processes in the bee body, suppressing their resistance, immunity, and metabolism (Ptaszyńska et al., 2018; Neov et al., 2019; Almasri et al., 2021). Therefore, modern beekeeping creates the necessary conditions to increase bee viability, health, productivity, and reproduction (Hrechka & Senchylo, 2022).

In the domestic and foreign practice of beekeeping, artificial feeding of bees is used with new effective means of natural origin and certain mineral elements as metabolic stimulants of organic and inorganic origin. These substances and compounds added in different doses to feeding can affect physiological and biochemical processes and increase the productivity and resistance of honey bees (Dvylyuk & Kovalchuk, 2017; Romaniv et al., 2018; Tauber et al., 2019).

Preparations of natural origin can avoid many negative phenomena since their mechanism of action activates the body's natural protective properties. Probiotic preparations in beekeeping, which hurt pathogenic microflora and promote the development of beneficial microflora in bees' gastrointestinal tracts, are relevant in this direction.

The gut microbiota of honey bees consists of 8—10 bacteria genera, which comprise more than 97% of the entire community (Kwong & Moran, 2016). Most bacterial genera include closely related species with a high level of strain diversity, the most common of which are genera of lactic acid bacteria Lactobacillus (Bombilactobacillus) (Zheng et al., 2020), as well as Gilliamella, Snodgrassella, and Bifidobacterium. Bee gut bacteria are adapted to their diverse food niches, play an essential role in digestive processes, and are beneficial for the host's lipid and mineral nutrition, immune homeostasis, and resistance to pathogens (Zheng et al., 2018; Kovalshuk et al., 2021; Lazarenko et al., 2021). A recent study found that oral supplementation with bee gut Lactobacillus increased hemolymph glycerophospholipids and improved memory in bumblebees (Li et al., 2021).

At the same time, it has been proven that the vitality and resistance of the honey bee organism largely depend on mineral nutrition, which affects metabolic processes at the level of tissues, organs, and systems (Dvylyuk & Kovalchuk, 2017; Kovalchuk et al., 2020). The use of biotic

trace elements in bee feeding as highly active compounds produced by nanotechnology participate in protein, lipid, carbohydrate, and mineral metabolism, activate enzyme systems, etc. (Kovalchuk et al., 2014; Dvylyuk & Kovalchuk, 2017; Cho et al., 2020).

The influence of various amounts of mineral and organic compounds obtained based on nanotechnological citrates on the metabolic processes of the bees' bodies was clarified. Several works were published based on the research results (Kovalchuk et al., 2014; Dvylyuk & Kovalchuk, 2017). A higher biological efficiency of adding nanocarboxylates of biotic elements than their mineral salts in bee feeding was established (Kovalchuk et al., 2021).

Adding some aspects to bee feed as metabolic stimulators introduced in different doses affects the correction of physiological and biochemical processes and increases their productivity and resistance (Dvylyuk & Kovalchuk, 2017). Such mineral components include Co, Ge, Se, Cr, Ni, and others. The indicated results, as well as previous studies of the IBT of NAS of Ukraine using citrates of certain microelements and probiotics (Romaniv et al., 2018; Kovalchuk et al., 2019), provide a theoretical basis for the development of new nano- and biotechnological means and drugs to increase the resistance and reproduction of bees. However, the biological effect of the newly synthesized nanotechnological Ge citrate mineral complex in combination with probiotic preparations of the *L. casei* B-7280 class has not been studied to date.

In connection with the earlier research, the **purpose** of this work was to study the influence of the lyophilized probiotic strain *Lactobacillus casei* IMV B-7280 in combination with nanotechnological Ge citrate on changes in the phospholipid composition of bee body tissues in laboratory conditions.

Materials and Methods. *Experimenting*. The research was conducted on the Carpathian breed honey bees from the laboratory apiary-vivarium of the Institute of Animal Biology of NAS of Ukraine. Lyophilized probiotic strain *Lactobacillus casei* IMV B-7280 was used in the research. This strain was isolated in the Department of Problems of Interferon and Immunomodulators of D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine (IMV) from the associated culture of biological material and deposited in the Ukrainian collection of microorganisms of the IMV. Before each experiment, the viability of the lyophilized strains was checked by monitoring their growth on Mann-Rogose-Sharpe (MRSA) medium at 37 °C for 24—48 h.

The research was conducted under laboratory thermostat conditions on three bee colonies similar in weight, colony strength, and queen age. 50—60 bees were selected and formed into three groups. Bees of the control and research groups were kept in cages-containers with a volume of 4 dm^3 in similar conditions to a TS-80M-3 laboratory thermostat with micro ventilation at 30 °C and humidity of 74—76%.

Bees of the control group (C) were grouped by feeding with 60% sugar syrup (SS) in the amount of 1 cm^3/group/day. Experimental group 1 of bees (E 1), in addition to 1 cm^3 of sugar syrup, received 0.1 μg of Ge in the form of nanotechnological citrate (NTC) (Kosinov & Kaplunenko, 2009) and a solution of the probiotic *L. casei* B -7280 at a concentration of 10^6 CFU/cm^3; experimental group 2 of bees (E 2), in addition to 1 cm^3 of sugar syrup, received 0.2 μg of Ge in the form of citrate and immunobiotic *L. casei* B-7280 at a concentration of 10^6 CFU/cm^3.

Drinking SS, Ge citrate, and probiotics took 34 days. During the preparatory period and on the 34th day of the experimental period, live bees were selected from the control and experimental groups for physiological and biochemical studies to determine the content of total phospholipids and the ratio of their classes in tissue homogenates of the entire organism.

Homogenized tissue (1g) was extracted with 20 cm^3 of a mixture of chloroform-methanol in a ratio of 2:1 (v/v) according to Folch's method

(Folch et al., 1957). The mixture was filtered through a deashed filter, a blue band. 4 cm^3 of an aqueous solution of 0.74% KCl was added to each sample of lipid extract. After 24 hours, the upper phase containing hydrophobic peptides was removed with a water pump, and the lower phase containing lipids was used in further studies.

The content of total phospholipids was determined by the amount of inorganic phosphorus in the lipid extract, as described in (Vaskovsky et al., 1975), and their mass was calculated in mg/g of tissue.

Separation of phospholipids. To separate phospholipids by one-dimensional thin-layer chromatography on silica gel (L 5/40μ, LSL 5/40μ, Chemapol, Czech Republic), a solvent system of chloroform—methanol-water in the ratio 65:25:4 (v/v/v) was used (Kates, 1986). Crystalline iodine vapor was used as a developer. The developed plates were scanned on HP Deskjet 2050 (China). The identification of individual subclasses of phospholipids was carried out using Rf values. Phospholipid subclasses were quantified using the TotalLab TL120 software (Nonlinear Dynamics Limited, UK) and expressed as a percentage of total content.

Study of the spectrum of the intestinal microbiome. To determine the qualitative and quantitative spectra of the intestinal microbiota of bees, on the 34th day, the midgut and hindgut (separately) were taken from the bees of either experimental group. The obtained samples were placed in microtubes of the «Eppendorf» type, weighed, filled with 1 mL of physiological solution, and homogenized in a sterile mortar with sterile sand. The resulting suspension was diluted to concentrations of 10^{-5} and 10^{-7} through a series of tenfold dilutions with sterile 0.15 M NaCl, and 100 μL was taken for vaccination of solid selective nutrient media for the cultivation of various groups of microorganisms:

- meat-peptone agar (MPA) — a medium for isolation and cultivation of aerobic and facultatively anaerobic microorganisms;
- BAIRD-PARKER-Agar («Merck,» Germany) — a selective medium for the isolation of staphylococci;
- KF-Streptococcus agar («Merck,» Germany) — a selective medium for isolation of streptococci;
- Man-Rogosa-Sharpe agar (MRSA, HiMedia, India) — a selective medium for isolation of lactobacilli;
- Bifidum agar (BA, HiMedia, India) — a selective medium for isolation of bifidobacteria;
- ENDO (HiMedia, India) — a selective medium for isolation of coliform bacteria;
- Saburo (HiMedia, India) — a selective medium for isolation of microscopic fungi;
- Pseudomonas agar (HiMedia, India) — a selective medium for isolation of pseudomonads.

Plates were incubated under appropriate conditions, and colonies of typical morphology were counted for each group of microorganisms. The data were expressed as Lg colony-forming units (CFU) in 1 mg of the studied sample.

The research was conducted following the «General Ethical Principles of Animal Experiments» (VII National Congress of Bioethics, Kyiv, 2019) and the European Convention on the Protection of Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

Statistical analysis. All obtained digital data were processed using the STATISTICA computer program, which used variational statistics, and the Excel program from Microsoft Office in 2007 and 2010. Numerical data are presented as arithmetic mean (M) and standard error (± m). Differences between groups were considered statistically significant at $p < 0.05$.

Results. Phospholipids take an active part in the formation of the lipid bilayer of biomembranes, affect the biochemical mechanisms of temperature adaptation, maintain the microviscosity of membranes, including several metabolic functions, in particular, enzyme catalysis reactions, ion transport, and intracellular signaling, and they are also involved in receptor-mediated

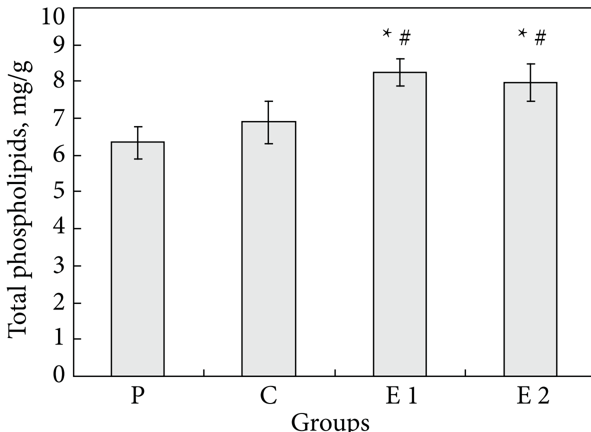

Fig. 1. The content of total phospholipids in the body tissues of bees: P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05 — probable differences between the preparatory and experimental periods by groups; # p<0.05 — probable differences between the control and experimental groups

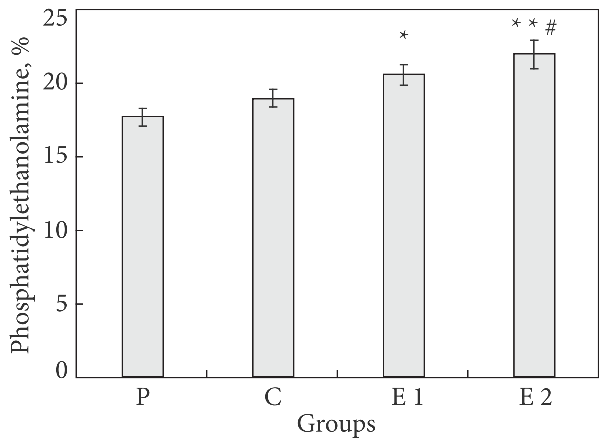

Fig. 2. The content of phosphatidylethanolamine in homogenates of bee body tissues (%, M±m, n=5): P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05; ** p<0.01 — probable differences between the preparatory and experimental periods by groups; # p<0.05 — probable differences between control and experimental groups

endocytosis of food components in the animal body (Romaniv et al., 2018; Li et al., 2021; Dai et al, 2021). In the homogenates of bee body tissues, an increase in the content of total phospholipids in E 1 and E 2 groups was found, respectively, by 30.28% (p<0.01) and 25.87% (p<0.05) compared to the preparatory period (Fig. 1).

In the subclasses of phospholipids, 17.66—21.90% of phosphotidylethanolamine (PhEA), 12.23—14.96% of phosphatidylinositol (PhI), 23.79—28.45% of phosphatidylcholine (PhH), 14.66—15.24% of phosphatidylserine (PhS), and 11.84—15.78% of sphingomyelin (SM) were found along with 10.65 —13.68% of lysophosphatidylcholine (LFH). In the fractional composition of phospholipids, an increase in the content of phosphatidylethanolamine was established in bees of groups E 1 and E 2 by 16.14% (P<0.05) and 24.01% (P<0.01) concerning the preparatory period, and group E 2 by 15.69% (P<0.05) compared to the control group (Fig. 2).

A decrease in the content of phosphatidylinositol was established in group E 2 by 11.70% (p<0.05) before the preparatory period and by

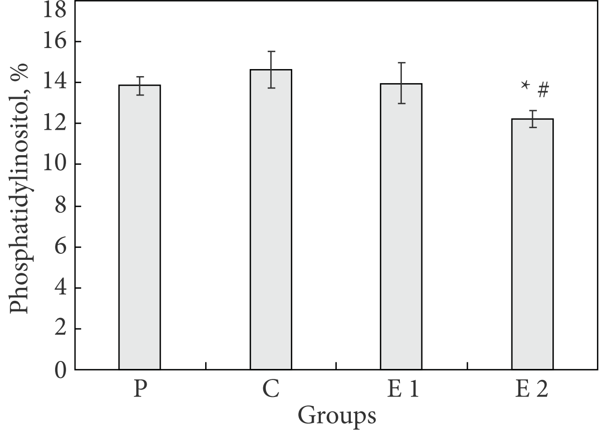

Fig. 3. The content of phosphatidylinositol in homogenates of bee body tissues (%, M±m, n=5). P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05 — probable differences between the preparatory and experimental periods by groups; # p<0.05 — probable differences between control and experimental groups

16.40% (p<0.05) compared to the control group of bees (Fig. 3).

An increase in phosphatidylcholine was observed in bees of the E 1 and E 2 groups by

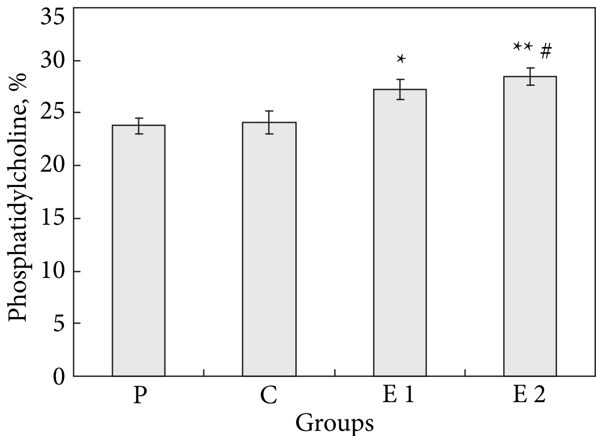

Fig. 4. The content of phosphatidylcholine in homogenates of bee body tissues (%, M±m, n=5). P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05; ** p<0.01 — probable differences between the preparatory and experimental periods by groups; # p<0.05 — probable differences between control and experimental groups

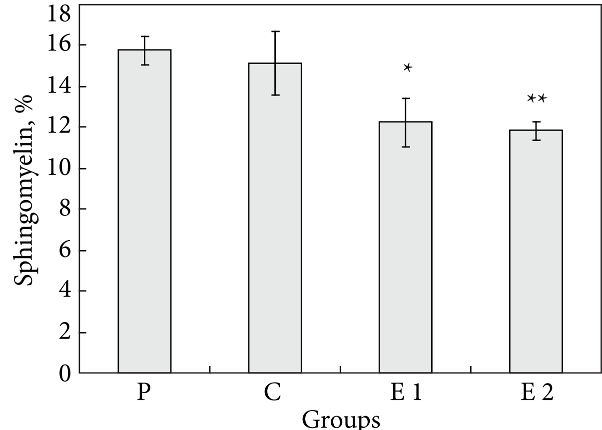

Fig. 5. The content of sphingomyelin in homogenates of bee body tissues (%, M±m, n=5). P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05; ** p<0.01 — probable differences between the preparatory and experimental periods by groups.

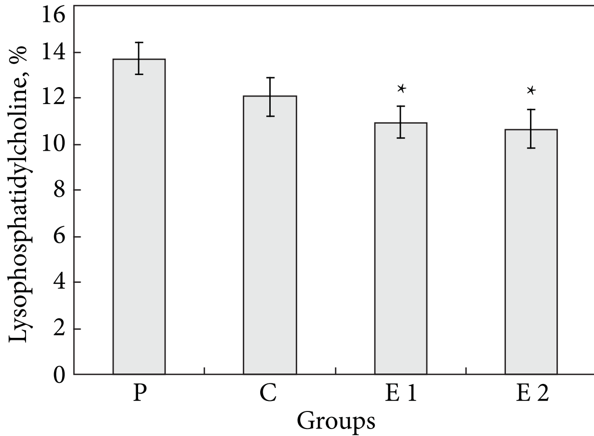

Fig. 6. The content of lysophosphatidylcholine in homogenates of bee body tissues (%, M±m, n=5). P — preparatory period, C — control group, E 1 — first experimental group, E 2 — second experimental group. * p<0.05 — probable differences between the preparatory and experimental periods by groups

14.54% (p<0.05) and 19.59% (p<0.01) according to the preparatory period and by 18.15% (P<0.05) in E 2 compared to the control group (Fig. 4).

The content of sphingomyelin decreased in the bees of groups E 1 and E 2 by 22.37% (p<0.05) and 24.97% (p<0.01) compared to the preparatory period (Fig. 5).

A decrease in the content of lysophosphatidylcholine in the bees of groups E 1 and E 2 was established to be by 20.10% and 22.15% (p<0.05) compared to the preparatory period (Fig. 6).

As for phosphatidylserine, its content in the body tissues of bees had no significant changes in the experimental groups compared to the preparatory period and the control group of bees, and its differences are improbable.

In the midgut of bees, aerobic and facultatively anaerobic microorganisms decreased only in group E2, whereas staphylococci decreased separately in both experimental groups (Table 1).

On the 34th day of the experiment, the number of streptococci and coliform bacteria in the midgut of bees remained at the control level in both experimental groups.

Microscopic fungi and pseudomonads were utterly eliminated from the midgut of bees for 34 days under the conditions of application of *L. casei* IMV B-7280 in the amount of 10^6 CFU + Ge in the form of citrate.

On the 34th day of the study, both experimental groups exhibited an increase in the number of lactobacilli and bifidobacteria in the midgut of bees.

In the hindgut, the number of aerobic and facultatively anaerobic microorganisms and staphylococci was lower than in the control in both experimental groups (Table 2). In contrast, streptococci and coliform bacteria remained at the control level on day 34 in E 1 and E 2.

We also noted that in the hindgut of bees that received supplementary feeding in the form of 60% sugar syrup 1 mL/group/day + *L. casei* IMV B-7280 in the amount of 10^6 CFU + 0.1 μg of Ge in the form of citrate for 34 days (E 1), the number of microscopic fungi and pseudomonads decreased significantly. On the other hand, in E 2, which received the double dose of Ge in the form of citrate for 34 days, these opportunistic microorganisms were eliminated, which may indicate a possible direct or indirect antimicrobial effect of Ge citrate.

After 34 days of the experiment, the number of lactobacilli and bifidobacteria in the hindgut of bees that received the probiotic strain *L. casei* IMV B-7280 and Ge citrate was higher.

Discussion. Cell membrane lipids, mainly phospholipids, form a bilayer that acts as a barrier between the cell and the environment and between different cell organelles. Numerous studies show that the lipid bilayer not only functions as a structural barrier but also plays a crucial role in the regulation of many cellular processes due to the diversity of membrane lipids (Wu et al., 2016; Sunshine & Iruela-Arispe, 2017; Harayama & Riezman, 2018). Analysis of the obtained data shows that the addition of different doses of nanotechnological Ge citrate and the probiotic strain *L.*

Table 1. **Spectrum of midgut microbiota of bees fed with *L. casei* strain IMV B-7280 and Ge citrate**

Group	The number of microorganisms sown on nutrient media (Lg CFU/mg)							
	MPA	BAIRD-PARKER-Agar	KF-Streptococcus agar	ENDO	Saburo	Pseudomonas agar	MRSA	BA
Control	4.12 ± 0.12	3.55 ± 0.09	3.14 ± 0.04	3.86 ± 0.14	3.27 ± 0.21	2.95 ± 0.02*	2.83 ± 0.09	2.37 ±0.05
Group E 1	3.72 ± 0.06	2.18 ± 0.04**	3.10 ± 0.07	4.25 ±0.15	0**	0**	4.12 ± 0.04**	3.17 ± 0.03**
Group E 2	3.12 ± 0.04*	2.06 ± 0.06**	2.87 ± 0.11	4.11 ±0.21	0**	0**	3.98 ± 0.08**	3.22 ± 0.02**

*p < 0.05; **p < 0.01 compared to control

Table 2. **Spectrum of hindgut microbiota of bees fed with *L. casei* strain IMV B-7280 and Ge citrate**

Group	The number of microorganisms sown on nutrient media (Lg CFU/mg)							
	MPA	BAIRD-PARKER-Agar	KF-Streptococcus agar	ENDO	Saburo	Pseudomonas agar	MRSA	BA
Control	4.93 ± 0.05	4.18 ± 0.04	3.27 ± 0.05	4.51 ± 0.07	3.78 ± 0.04*	4.66 ± 0.08*	3.52 ± 0.09	3.12 ± 0.11
Group E1	4.01 ± 0.06*	2.33 ± 0.05**	2.99 ± 0.17	4.65 ± 0.14	1.55 ± 0.09**	1.20 ± 0.12**	5.78 ± 0.05**	4.62 ± 0.06**
Group E2	3.55 ± 0.03**	2.12 ± 0.08**	3.17 ± 0.08	4.87 ± 0.10	0**	0**	5.06 ± 0.10**	4.33 ± 0.04**

*p < 0.05; **p < 0.01 compared to control

casei B-7280 to sugar syrup affected both the total content of phospholipids (Fig. 1) and the ratio of their subclasses. An increase in the content of total phospholipids in the tissues of bees of groups E 1 and E 2 compared to the control group and the preparatory period may indicate the stimulating effect of the probiotic *L. casei* in combination with NTC Ge on the synthesis of these lipids in the body of bees and their adaptive capacity.

Phosphatidylethanolamine (PhEA) is one of the most common phospholipids in the body. It is part of the cell membrane and contains many unsaturated fatty acids, a source of their active metabolites. PhEA is involved in signal transduction as a substrate of phospholipase D (Braun et al., 2016) of the ethanolamine component of glycosylphosphatidylinositol anchors that bind to proteins on the surface of the cell membrane and perform a signaling function (Dai et al., 2021).

The determined increase in the content of PhEA (Fig. 2) can be interpreted as maintaining its homeostasis and inhibiting phospholipase D in the body of bees under the action of the probiotic *L. casei* in combination with the applied doses of Ge citrate. Some studies have shown that increasing the content of PhEA by adding its precursor ethanolamine to food or overexpressing the phosphatidyl biosynthetic enzymes phosphatidylserine decarboxylase (PSD) extends the lifespan of yeast organisms as well as insects and mammals (Rockenfeller et al., 2015; Dai et al., 2021). The lifespan extension effect of PhEA is associated with an increase in the autophagic flux (Rockenfeller et al., 2015), a positive regulator of lifespan in many studied organisms (Hansen et al., 2018). It has been suggested that adding PhEA may increase lifespan by promoting autophagy. In addition, reducing PhEA by inhibiting PSD encourages the production of reactive oxygen species (ROS) and accelerates aging in yeast (Rockenfeller et al., 2015). The link between PhEA and ROS is also supported by *C. elegans* studies showing that PhEA supplementation increases the resistance to oxidative stress and promotes longevity via DAF-16 (Park et al., 2021). These studies suggest that PhEA plays a crucial role in prolonging life by acting as a regulator of ROS production. Therefore, PSD-mediated synthesis of PhEA, which occurs in the mitochondrial inner membrane, is essential for the electron transport chain activity (Calzada et al., 2019). Thus, it can be assumed that the increase in the content of PhEA in the body of bees of the experimental groups when the *L. casei* probiotic is added to the SS in combination with the applied doses of Ge citrate causes the activation of mitochondria and, accordingly, affects the life span.

Phosphatidylinositol is a smaller fraction of cellular phospholipids than PhEA. Still, it controls almost all aspects of cell life and death and is a crucial signaling element in the cells of living organisms. It can be hydrolyzed to release 1,2-diacylglycerol and inositol-1,4,5-triphosphate, which in animal cells lead to activation of protein kinase C and cellular calcium mobilization, respectively. Under the action of probiotic *L. casei* and Ge citrate in a dose of 0.2 μg, a decrease in the content of PHI was observed in group E 2 (Fig. 3). It is known that phosphoinositols are involved in signal transduction processes and are a source of such vital messengers as diacylglycerol, inositol phosphates, and arachidonic acid (Dickson & Hille, 2019; Blunsom & Cockcroft, 2020). Based on the above, the detected changes in the content of phosphatidylinositol can be explained by the inhibition of the activity of phospholipase C. The basis of the changes may be a decrease in the rate of receptor-mediated hydrolysis of phosphatidylinositol by phospholipase C (Blunsom & Cockcroft, 2020). The reduction in the content of PhI in the phospholipids of group E 2 may be a consequence of the activation of phospholipase C by Ge ions as a specific adaptive response to the action of this element.

The determined increase in the content of phosphatidylcholine (Fig. 4) in the lipids of bee tissues under the action of the probiotic *L. casei* IMV B-7280 in combination with Ge citrate may

be due to the effect on the inhibition of phospholipase D. This enzyme catalyzes its hydrolysis with the formation of phosphatidic acid. PhH homeostasis is critical for organelle functions, while its reduction shows cellular stress, known as lipid bilayer stress (Halbleib et al., 2017; Shyu et al., 2019). Thus, the cell develops an adaptive mechanism whereby the loss of PhH affects multiple cellular processes through the stress response (Koh et al., 2018; Ho et al., 2020). Furthermore, an increase in PhH may prolong the bees' life span. Some studies report changes in PhH content with animal age, taking into account species and tissue specificity. Thus, PhH content is markedly reduced in old nematodes (Gao et al., 2017; Wan et al., 2019) and shows a significant decrease in the kidneys of old mice (Braun et al., 2016). This also applies to humans, as PhH content is higher in centenarians than in the elderly (Montoliu et al., 2014).

Shingomyelin (ceramide) is an essential structural component of biological membranes and one of the endpoints of sphingolipid synthesis. Along with phosphatidylcholine, CM is one of the most common phospholipids in biological membranes. Structural diversity and cellular topology allow ceramide to exert multiple effects and be metabolized into other bioactive sphingolipids. Some diseases (cancer, inflammation, atherosclerosis, diabetes, and some rare diseases) involve the sphingomyelin cycle in the body. The type and composition of sphingolipids modulate the biophysical properties of membranes, which can be organized into two-dimensional domains. Membrane properties determined by the specific type and amount of sphingolipids allow biological membranes to adapt to temperature, pH, and membrane tension (Sessa et al., 2021; Trenti et al., 2022). For example, the presence of SM increases the stiffness and compactness of the plasma membrane (PM). In mammalian membranes, CMs with different acyl chains, unsaturated phospholipids, and cholesterol can be used by the cell to improve the lateral structure of membranes (Sessa et al., 2021).

Lysophosphatidylcholine is a phospholipid component of oxidized low-density lipoproteins (Ox-LDL). This subclass of phospholipids originates from the cleavage of phosphatidylcholine by phospholipase A 2 and is catabolized to other substances by various enzymatic pathways. LPHH exerts pleiotropic effects mediated by its receptors, G protein-coupled signaling receptors, Toll-like receptors, and ion channels to activate multiple secondary messengers (Law et al., 2019; Ren et al., 2022). The established reduction of LPHH (Fig. 6) in the body of bees of groups E 1 and E 2 under the action of probiotic *L. casei* B-7280 in combination with different doses of Ge citrate compared to the control group can be explained by the inhibitory effect phospholipase A 2 on the cleavage of phosphatidylcholine.

Phosphatidylserine is one of the main phospholipids, which has the biochemical properties of an anionic phospholipid, binds to various proteins, and participates in many biological processes, such as enzyme activation, apoptosis, neurotransmission, and synaptic contraction (Ma et al., 2022).

PhS is formed by the exchange of head groups in the mammalian body cells with the help of PhS synthases; for example, PhS synthase 1 is responsible for the exchange of headgroup choline with PhH, and PhS synthase 2 is responsible for the exchange of headgroup ethanolamine with PhEA. Since PhS synthases 1 and 2 are regulated in the mitochondrial-associated membranes (MAMs) of the endoplasmic reticulum, PhS is produced in the endoplasmic reticulum and transported to the mitochondria or Golgi via the MAMs (Ma et al., 2022). In mitochondria, part of PhS is catalyzed to PhEA by PhS decarboxylase in the inner mitochondrial leaflet, while the other part of PhS is incorporated into the mitochondrial membrane. Under normal conditions, PhS is found exclusively in the cytoplasm of the plasma membrane, endoplasmic reticulum lumen, Golgi, mitochondria, and endosomes to support normal organelle function

(Kay & Fairn, 2019). It is located on the inner surface of the plasma membrane to maintain regular cellular activity. Flip-flopping of PhS to the outer surface of the bilayer can trigger apoptosis (Kiraz et al., 2016; Chua et al., 2019). Thus, adding Ge SS citrate and probiotic *L. casei* to the feed did not affect the relative content of PhS, which means the violation of asymmetry is essential in the functioning of membrane-bound enzyme systems.

In general, the detected changes in the lipid composition of the cell membranes of the body tissues of bees when the probiotic *L. casei* IMV B-7280 was added to the sugar syrup in combination with different doses of NTC Ge may be a consequence of their multifactorial influence on the structure and function of individual tissues and organs.

Also, the shift in the spectrum of different fractions of phospholipids toward smaller contents of difficult-to-oxidize lysophosphatidylcholine and sphingomyelin with increasing easy-to-oxidize phosphatidylcholine and phosphatidylethanolamine may indicate the stabilization of compensatory mechanisms for supporting cell membranes.

As known, the interaction between microbiota and bee organism is common among pollinating insects. The gut microbiome of the honey bee actively participates in protection against infections and degradation of pollen coat polysaccharides, as well as in the detoxification of pollutants and toxic plant compounds. In addition, the honey bee microbiome is essential for honey production and Perga during maturation (Tsadila et al., 2023).

In bees, the symbiotic intestinal microflora is essential not only for digestion but also for the antagonistic activity against pathogenic microorganisms and the functioning of the body's immune system. Probiotic supplementation is essential when bees limit contact with the environment and natural probiotic bacteria (Fedoruk et al., 2023).

Using *Lactobacillus casei* strain B-7280 and Ge citrate increased the number of lactobacilli and bifidobacteria in both parts of the intestine and decreased the number of staphylococci, streptococci, and microscopic fungi.

Thus, it can be considered appropriate to continue research on probiotic lactobacilli strains to create a complex preparation with Ge citrate to increase bees' life expectancy and honey productivity. This preparation will also support the homeostasis of their microbiome, which will provide natural protection for the bees' bodies and maintain homeostasis.

Conclusions. 1. In the doses applied, nanotechnological Ge citrate and *L.casei* show a dose-dependent biological effect on honey bees fed SS in a laboratory thermostat for 34 days.

2. The use of NTC Ge and *L. casei* in the feeding of bees leads to an increase in the absolute content of total phospholipids ($p<0.05$) in the body of bees under the action of 0.1 and 0.2 μg of Ge compared to the preparatory period and the control group, which may be due to the influence of these additives on the level of the ratio of individual subclasses of phospholipids.

3. Supplementation of bees with NTC Ge at 0.1 and 0.2 μg/mL SS and 10^6 CFU/mL SS *L. casei* was characterized by differences in the distribution of individual classes of phospholipids in bee tissue homogenates with a higher relative content of phosphatidylethanolamines and phosphatidylcholines and a lower content of sphingomyelin and lysophosphatidylcholine in groups E1 and E2 compared to the control group, but a higher content of phosphatidylethanolamines and phosphatidylcholines and a lower content of phosphatidylinositols in group E2 bees concerning the preparatory period, which indicates a dose-dependent effect of these additives on the metabolism of lipids and their fractions.

4. The use of *Lactobacillus casei* B-7280 and Ge citrate for feeding bees under the conditions of a laboratory thermostat led to quantitative

changes in the composition of the intestinal microbiota of bees, in particular, an increase in the number of lactic acid bacteria and bifidobacteria and a decrease in the number of some other groups of microorganisms in the intestine.

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Compliance with ethical principles. None of the experiments described in this article involved using vertebrate animals.

Conflict of interest. The authors declare no conflict of interest.

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А.З. Пилипець[1], І.І. Ковальчук[1,2], Л.П.Бабенко[3], Р.С. Федорук[1],
Т.М. Химинець[2], М.М. Цап[1], М.М. Романович[1], Р.Л. Андрошулік[1], Т.В. Жиліховська[3]

[1] Інститут біології тварин НАН України,
вул. Василя Стуса, 38, Львів, 79034, Україна

[2] Львівський національний університет ветеринарної медицини та біотехнологій імені С.З. Ґжицького,
вул. Пекарська, 50, Львів, 79010, Україна

[3] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна

ВПЛИВ РІЗНИХ ДОЗ GE ЦИТРАТУ ТА ПРОБІОТИКА *LACTOBACILLUS CASEI* B-7280
НА ФОСФОЛІПІДНИЙ СКЛАД ТКАНИН ОРГАНІЗМУ БДЖІЛ

Мета. Дослідити в лабораторних умовах зміни фосфоліпідного складу тканин організму бджіл за впливу ліофілізованого пробіотичного штаму *Lactobacillus casei* IMV B-7280 у поєднанні з нанотехнологічним Ge цитратом. **Методи.** Дослідження проведено на медоносних бджолах карпатської породи. Бджоли контрольної групи отримували підгодівлю з 60 % цукрового сиропу в кількості 1 см^3/групу/добу. Експериментальна група бджіл 1 (Е 1), додатково до 1 см^3 цукрового сиропу, отримувала 0,1 мкг Ge у вигляді нанотехнологічного цитрату і розчин пробіотика *L. casei* B-7280 у концентрації 10^6 КУО/см^3; експериментальна група Е 2 — додатково до 1 см^3 цукрового сиропу додавали 0,2 мкг Ge у вигляді цитрату і *L. casei* B-7280 у концентрації 10^6 КУО/см^3. Тривалість випоювання цукрового сиропу, Ge цитрату та пробіотика — 34 дні. У підготовчий період і із завершенням дослідного періоду з контрольної та експериментальних груп відбирали живих бджіл для проведення фізіолого-біохімічних досліджень з визначенням вмісту загальних фосфоліпідів і співвідношення їх класів у гомогенатах тканин всього організму. Вміст загальних фосфоліпідів визначали за кількістю неорганічного фосфору в ліпідному екстракті. Для розділення фосфоліпідів використовували тонкошарову хроматографію на силікагелі. Ідентифікацію окремих фосфоліпідів проводили за величинами Rf. Кількісний аналіз підкласів фосфоліпідів проводили за допомогою програмного забезпечення TotalLab і виражали у відсотках від загального вмісту. **Результати.** Результати досліджень показали, що в гомогенатах тканин організму бджіл збільшується вміст загальних фосфоліпідів порівняно до підготовчого періоду. У фракційному складі фосфоліпідів встановлено збільшення вмісту фосфатидилетаноламіну і фосфатидилхоліну у тканинах бджіл груп Е 1 та Е 2 відносно підготовчого періоду. Відзначено збільшення вмісту фосфатидилетаноламіну і фосфатидилхоліну та зменшення фосфатидилінозитолу у ліпідах тканин організму Е 2 групи порівняно до контрольної групи. Також встановлено зменшення вмісту фосфатидилінозитолу у тканинах бджіл Е 2 групи відносно підготовчого періоду. Вміст сфінгомієліну і лізофосфатидилхоліну зменшувався у ліпідах тканин бджіл Е 1 та Е 2 груп відносно підготовчого періоду. Застосування штаму *Lactobacillus casei* B-7280 і цитрату Ge приводило до збільшення кількості лактобацил та біфідобактерій в обох відділах кишківника, а також до зниження кількості стафілококів, стрептококів та мікроскопічних грибів. **Висновки.** Нанотехнологічний цитрат Ge і пробіотик *L. casei* у застосованих дозах за умов підгодівлі їх цукровим сиропом у лабораторному термостаті впродовж 34 діб виявляють дозозалежну біологічну дію в медоносних бджіл підвищенням вмісту загальних фосфоліпідів і змінами співвідношення окремих підкласів фосфоліпідів. Проте також вказує на зміщення спектра різних фракцій фосфоліпідів до зменшення вмісту вжкоокиснюваних (лізофосфатидилхоліну та сфінгомієліну) зі збільшенням легкоокиснюваних (фосфатидилхоліну, фосфатидилетаноламіну), що може свідчити про стабілізацію компенсаторних механізмів підтримки клітинних мембран. Застосування *Lactobacillus casei* B-7280 і цитрату Ge для підгодівлі бджіл за умов лабораторного термостату приводило до кількісних змін у складі кишкової мікробіоти бджіл, зокрема до збільшення кількості молочнокислих бактерій та біфідобактерій, а також зменшення кількості деяких інших груп мікроорганізмів в кишківнику.

Ключові слова: *медоносні бджоли, тканини організму, Ge цитрат нанотехнологічний, пробіотик, фосфоліпіди.*

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O.V. GUDZENKO[1]*, L.D. VARBANETS[1], V.O. IVANYTSIA[2]

[1] Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine

[2] Mechnikov Odesa National University,
2 Dvoryanska Str., Odesa, 65029, Ukraine

* Author for correspondence; e-mail: alena.gudzenko81@gmail.com

EFFECT OF CULTIVATION TEMPERATURE ON ELASTIC, FIBRINOGENOLITIC, AND COLLAGENASE ACTIVITY OF MARINE BACTERIA *BACILLUS ATROPHAEUS* 08, *BACILLUS LICHENIFORMIS* 043, AND *BACILLUS SUBTILIS* 248

Marine microorganisms are the main suppliers of vital organic compounds in the complex ecosystem of the World Ocean thanks to the expression of a wide range of unique enzymes, including proteases. The activity of proteases depends on both the taxonomic affiliation of the strain and the source and place of isolation of the microorganism. Environmental factors of bacteria, such as water salinity, its temperature, pressure, and illumination, largely determine both the composition and the physicochemical properties and substrate specificity of the enzymes produced by them. Since not only the temperature of the residence of microorganisms but also the temperature of their cultivation can significantly affect the activity, the ***purpose*** *of the work was to determine the optimal cultivation temperatures of producers, necessary to achieve the maximum elastase, fibrinogenolytic, and collagenase activities of the studied strains Bacillus atrophaeus 08, Bacillus licheniformis 043, and Bacillus subtilis 248, which were isolated from different depths of the Black Sea.* ***Methods****. The objects of research were three strains: Bacillus subtilis 08, Bacillus licheniformis 043, and Bacillus subtilis 248, which were isolated from bottom sediments* from *3 points at depths of 888—2080 m in the Black Sea. Cultures were grown at a temperature of 12, 28, and 42 °C with a rotation speed of 210 rpm for 5 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min. Methods of determining proteolytic (elastolytic, fibrinogenolytic, and collagenase) activity in the culture liquid supernatant were used.* ***Results.*** *Although the studied cultures were isolated from almost the same depth: Bacillus atrophaeus 08, Bacillus subtilis 248 (1499 m), and Bacillus licheniformis 043 (1537 m), the supernatants of their culture liquids showed different enzymatic activity depending on temperature and growth dynamics. So, for Bacillus atrophaeus 08, the highest elastase and collagenase activities were detected at 28 °C on the second day of cultivation, while fibrinogenolytic activity was detected at 12 °C on the second day of cultivation. The maximum elastase and collagenase activites of Bacillus licheniformis 043 were manifested at 28 °C on the fourth and second day of cultivation, respectively. The highest fibrinogenolytic*

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activity was at 42 °C on the second day of cultivation. When studying Bacillus subtilis 248, it was shown that the maximum elastase activity is achieved at 12 °C on the second day of cultivation, the highest fibrinogenolytic activity was noted at 28 °C on the fourth day of cultivation, and to achieve maximum collagenase activity, it is necessary to grow at 42 °C for four days. ***Conclusions****. The temperature of cultivation of microorganisms plays a significant role in achieving maximum proteolytic activity. Choosing the optimal growing temperature allows for increasing elastase, fibrinogenase, and collagenase activities by several times. It was established that the dynamics of synthesis in different strains is significantly different.*

Keywords: *bacteria isolated from deep-sea sediments of the Black Sea, cultivation temperature, elastolytic, fibrinogenolytic, and collagenase activities.*

Marine microorganisms are the main suppliers of vital organic compounds in the complex ecosystem of the World Ocean thanks to the expression of a whole range of unique enzymes, including hydrolases (Mou & Zhu, 2022). Microbial hydrolases have long been used in various industries, and the rapid development of biotechnology has created a need for enzymes with new properties. The most well-studied class of hydrolases includes microbial proteases, which occupy a key position in terms of their commercial application. They play an important role in physiological processes due to their ability to hydrolyze various protein substrates. Thus, proteases are involved in such biological processes as blood clotting, control of cell death, and tissue differentiation. They catalyze a number of processes in tumor diseases and during infections caused by microorganisms and viruses. They are one of the three large groups of industrially important enzymes, accounting for about 60% of the worldwide sale of enzymes. So, they are widely used in various industries (meat processing, cheese making, cosmetology, detergents, etc.) and medicine (Song & Wei, 2023). The activity of proteases depends on both the taxonomic affiliation of the strain and the source and place of isolation of the microorganism. Environmental factors of bacteria, such as water salinity, temperature, pressure, and illumination, largely determine both the composition and physicochemical properties and substrate specificity of the enzymes produced by them. Since not only the temperature of the residence of microorganisms but also the temperature of their cultivation can significantly affect the activity, the **purpose** of the work was to determine the optimal cultivation temperatures of producers, necessary to achieve the maximum elastase, fibrinogenolytic, and collagenase activities of the studied strains *Bacillus atrophaeus* 08, *Bacillus licheniformis* 043, and *Bacillus subtilis* 248, which were isolated from different depths of the Black Sea.

Materials and Methods. The objects of research were 3 strains: *Bacillus subtilis* 08, *B. licheniformis* 043, and *B. subtilis* 248, which were isolated from bottom sediments from three points at depths of 888—2080 m in the Black Sea, from the horizons of cylindrical sediment cores with an interval of 5 cm. The samples from which the strains were identified were taken during the M84/2 expedition of the University of Bremen on the Meteor ship in March 2011 and transferred to Mechnikov Odesa National University (ONU) for microbiological research by Yu.P. Zaitsev and B.G. Aleksandrov (Institute of Marine Biology, NASU). Selected strains were identified previously (Ivanytsia & Ostapchuk, 2017) and are listed in Table 1.

The strains were grown and maintained on meat peptone agar slants by cultivating for 24 h at 28 °C.

For submerged fermentation, strains were cultivated in Erlenmeyer flasks containing 100 mL of medium of the following composition (g/L): KH_2PO_4 — 1.0; $MgSO_4x7H_2O$ — 0.75; $ZnSO_4xH_2O$ — 0.25; $(NH_4)_2SO_4$— 0.5; maltose — 1.0; gelatin - 10.0; yeast autolysate— 0.15; pH 7.0. Cultures were grown at a temperature of 12, 28, and 42 °C with a rotation speed of 210 rpm for 5 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min. Enzymatic activity was determined in the culture liquid supernatant.

The culture, grown in a medium of the above composition was used as an inoculum. The inoculum with the number of bacteria 10^4—10^5 cells/mL was added to the medium volume in the amount of 10%.

Elastase activity was determined colorimetrically by the color intensity of the solution during the enzymatic hydrolysis of elastin stained with Congo-rot using the Trowbridge et al. method (Trowbridge & Moon, 1972). The incubation mixture contained 5 mg of elastin, 2.0 mL of 0.01 M Tris-HCl buffer (pH 7.5) supplemented with 0.005 M $CaCl_2$, and 1 mL of test enzyme solution. The mixture was incubated for 5 h at 37 °C. Non-hydrolyzed elastin was separated by centrifugation at 8000g for 10 min. The color intensity was measured on an SF-26 spectrophotometer at 515 nm. The activity was calculated from a standard curve, which was obtained by measuring the color of the culture liquid supernatant from complete enzymatic hydrolysis of known amounts of elastin stained with Congo-rot. An activity unit was taken as the amount of enzyme that catalyzes the hydrolysis of 1 mg of the substrate within 1 min under standard conditions.

To determine fibrinogenolytic activity, fibrinogen was used as a substrate (Nidialkova & Chernyshenko, 2016). 1 mg of fibrinogen, 1.8 mL of Tris-HCl buffer (pH 7.5), and 0.2 mL of the studied preparation were added to the test sample incubated for 30—45 min at 37 °C. The reaction was stopped by adding 2 mL of 10% trichloroacetic acid (TCA) the control sample immediately. The samples were kept at room temperature for 20 min and then centrifuged at 10 000 g for 10 min to remove precipitated protein. Absorption was measured on an SF-26 spectrophotometer at a wavelength of 275 nm. The amount of enzyme that under the conditions of the experiment increases absorption by 0.01 within 1 min was taken as a unit of activity.

Collagenase activity was determined by the content of free amino acids in the reaction mixture in the reaction with ninhydrin (Moore & Stein, 1948). The unit of activity was the number of micromoles of released amino acids according to the standard curve constructed for leucine.

All experiments were performed in no less than 3—5 replications. Statistical processing of the results of the experimental series was carried out by standard methods using Student's t-test. The value of the null hypothesis $p < 0.05$ was taken as the critical level of reliability.

Results. The study of the influence of the cultivation temperature on the elastase activity of *Bacillus atrophaeus* 08 showed (Fig. 1 A) that it was noted from the very first day of cultivation at all temperatures studied but the highest activity was observed at 28 °C, regardless of the day of cultivation. The maximum elastase activity was 35.6 U/mL on the 2nd day of cultivation.

Fibrinogenolytic activity was also observed at all investigated temperatures during the entire cultivation period, but the optimal temperature for cultivation of *B. atrophaeus* 08 to achieve maximum fibrinogenolytic activity was 12 °C, after 3 days of cultivation (25.0 U/mL) (Fig. 1B). Moreover, it should be noted that at this cultivation temperature, starting from the 2nd day of cultivation, the fibrinogenolytic activity was higher than during cultivation at temperatures of 28 and 42 °C.

The study of the influence of cultivation temperature on the collagenase activity of *B. atrophaeus* 08 showed (Fig. 1 C) that it is insignificant, its maximum level (0.1 U/mL) is reached on the 2nd day of cultivation at 28 °C. This level of collagenase activity was maintained on the 3rd and 4th days of cultivation. On the 5th day,

Table 1. **Studied strains**

Strain	Station number, depth (m), horizon (cm)
Bacillus atrophaeus 08	242, 1499, 10—15
Bacillus licheniformis 043	233, 1537, 15—20
Bacillus subtilis 248	242, 1499, 15—20

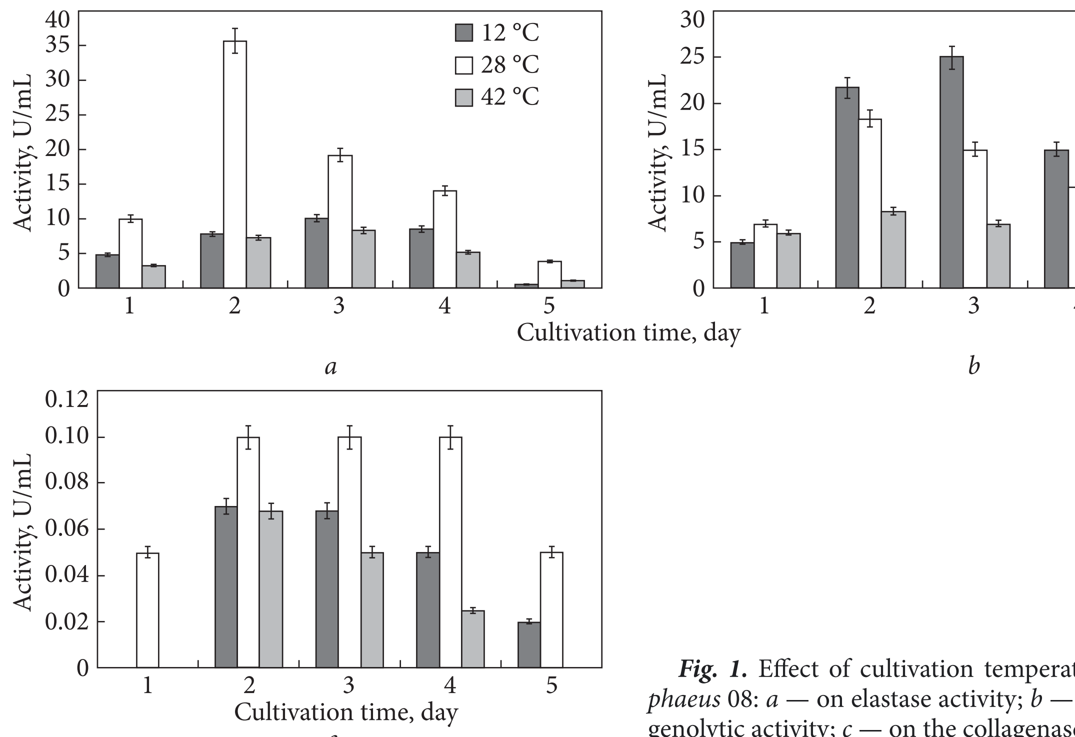

Fig. 1. Effect of cultivation temperature of *B. atrophaeus* 08: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

the activity decreased more than by 2 times and amounted to 0.05 U/mL.

The study of the influence of the growing temperature of *Bacillus licheniformis* 043 on the dynamics showed (Fig. 2 A) that the maximum elastase activity was observed at a temperature of 28 °C (18.85 U/mL) on the 4th day of cultivation. We consider as an interesting result the absence of elastase activity when cultivating the producer at 42 °C already on the 4th or 5th days. Significantly different results were noted for the fibrinogenolytic activity of *B. licheniformis* 043 (Fig. 2 B).

Thus, the greatest fibrinogenolytic activity was at a temperature of 42 °C throughout the entire cultivation period. Lowering the growing temperature of *B. licheniformis* 043 contributed to a significant decrease in activity. The maximum fibrinogenolytic activity, 41.66 U/mL, was on the 2nd day of cultivation at 42 °C, while at 28 °C it was 6 times lower (7.66 U/mL), and at 12 °C — more than 15 times and was only 2.66 U/mL.

The study of the level of collagenase activity of *B. licheniformis* 043 showed that its highest values were at 28 °C (Fig. 2 C).

On the 1st day of cultivation at this temperature, the activity was 0.25 U/mL. On the 2nd to 4th days of cultivation, it was at the same level — 0.52 U/mL. On the fifth day of cultivation, it slightly decreased and amounted to 0.35 U/mL. A slightly lower activity was observed at a cultivation temperature of 42 °C. At this temperature, the maximum collagenase activity, 0.42 U/mL, was reached on the 2nd day. Starting from the 3rd day of cultivation, it gradually decreased from 0.35 U/mL down to 0.1 U/mL on the 5th day of cultivation.

When cultivating *B. licheniformis* 043 at 12 °C, collagenase activity was not detected at all.

Studying the influence of the cultivation temperature on the elastase activity of *B. subtilis* 248 revealed (Fig. 3 A) that its maximum level, 18.6 U/mL, was reached at 12 °C on the 2nd day of cultivation.

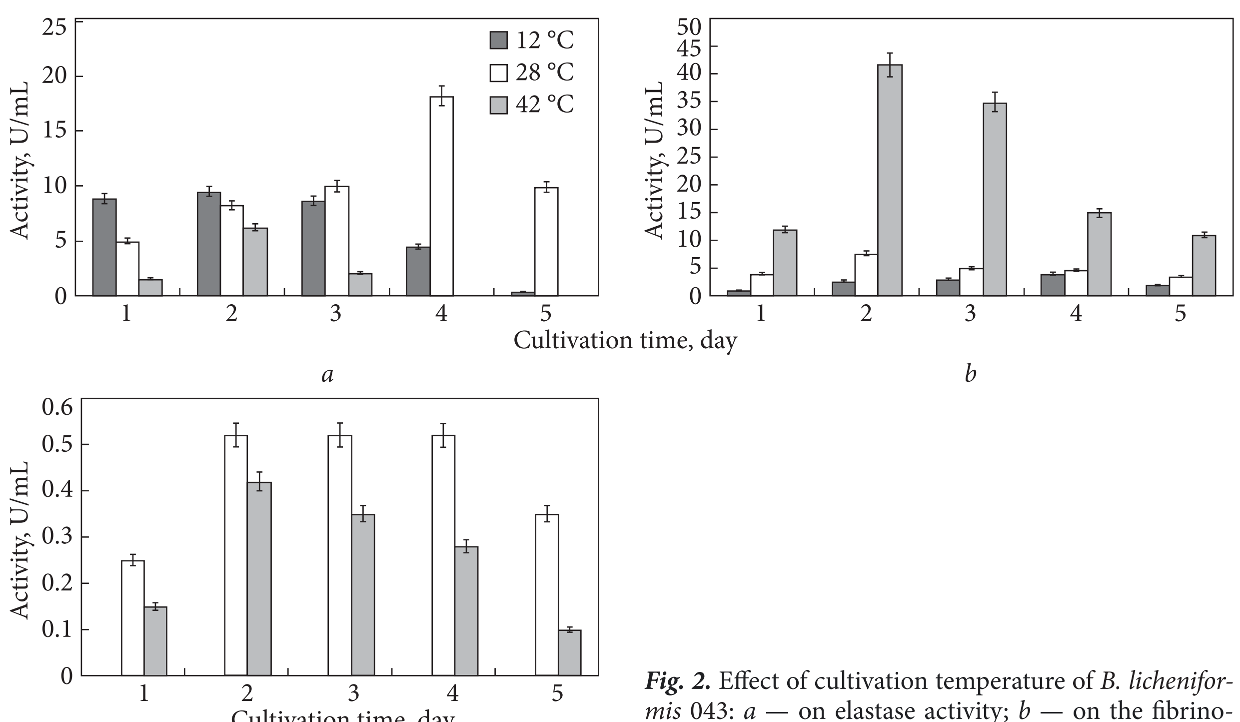

Fig. 2. Effect of cultivation temperature of *B. licheniformis* 043: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

Fig. 3 A. Effect of cultivation temperature of *B. subtilis* 248: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

Elastase activity was somewhat lower at this temperature for 1 day of cultivation (17.6 U/mL). With an increase in the time of cultivation of *B. subtilis* 248, a decrease in enzymatic activity was noted at 12 °C, with increasing the temperature of cultivation, the activity also increased, and at 42 °C for 3 days it amounted to 17.3 U/mL.

The study of the influence of the cultivation temperature on the fibrinogenolytic activity of *B. subtilis* 248 showed that a maximum activity was achieved at 28 °C (Fig. 3 B).

At this temperature, the highest fibrinogenolytic activity, 21.66 U/mL, was noted on the 4th day of cultivation, whereas on the 2nd and 3rd days of cultivation, it was almost at the same level — 21 U/mL. When growing at 42 °C, the maximum fibrinogenolytic activity, 16.3 U/mL, was already on the 2nd day of cultivation Starting from the 3rd day of cultivation, it decreased at this temperature.

When growing *B. subtilis* 248 at 12 °C, the activity was maximal on the 3rd day of cultivation (13 U/mL). An increase in the cultivation time contributed to a decrease in fibrinogenolytic activity.

To achieve the maximum collagenase activity of the *B. subtilis* 248 strain (Fig. 3 C), it is necessary to grow the strain for four days at 42 °C. Under these conditions, the activity was 0.95 U/mL.

At this cultivation temperature, activity was noted starting from the second day of cultivation. As shown in Fig. 3, on the 2nd day, it was 0.18 U/mL, on the 3rd and fifth days — 0.5 U/mL. It turned out to be interesting that on the first day of cultivation, collagenase activity was detected only at 12 °C.

Thus, the temperature of cultivation of microorganisms plays a significant role in achieving maximum proteolytic activity. Choosing the optimal growing temperature allows for an increase in elastase, fibrinogenase, and collagenase activities by several times. It was established that the dynamics of synthesis in different strains is significantly different.

Discussion. Each microorganism is characterized by an optimal growth temperature, so any temperature above or below this level worsens metabolic processes, as they slow down at such temperatures. In some microorganisms, the production of enzymes can be significantly reduced when grown at higher temperatures, because their denaturation is observed (Engqvist, 2018; Mehta & Sharma, 2016). At the same time, in some microorganisms, when they are grown at lower temperatures (Médigue et al., 2005), an increase in both the synthesis and activity of enzymes can be observed. Since the bacteria we studied were isolated from deep-sea sediments of the Black Sea, where the temperature is +5 — +9°C, we decided to investigate how the cultivation temperature can affect the activity of proteolytic enzymes. Although the studied cultures were isolated from almost the same depth: *Bacillus atrophaeus* 08 and *Bacillus subtilis* 248 at 1499 m, and *Bacillus licheniformis* 043 at 1537 m, the supernatants of their culture liquids showed different enzymatic activity depending on both temperature and growth dynamics. So, for *Bacillus atrophaeus* 08, the highest elastase and collagenase activities were detected at 28 °C on the 2nd day of cultivation, while fibrinogenolytic activity was detected at 12 °C on the 2nd day of cultivation. Elastase and collagenase activities of *Bacillus licheniformis* 043 were maximum at 28 °C on the 4th and 2nd day of cultivation, respectively. The highest fibrinogenolytic activity was at 42 °C on the second day of cultivation. When studying the temperature of cultivation of *Bacillus subtilis* 248, it was shown that the maximum elastase activity is achieved at 12 °C on the 2nd day of cultivation, the highest fibrinogenolytic activity was noted at 28 °C on the 4th day of cultivation, and to achieve maximum collagenase activity, it was necessary to grow at 42 °C for four days.

Thus, based on the conducted research, it is possible to mark the most effective producers of elastase — *Bacillus atrophaeus* 08 and fibrinoge-

nase — *Bacillus licheniformis* 043. If the elastolytic activity of *Bacillus atrophaeus* 08 is 35.6 U/mL, then the elastase activity of the culture liquid supernatant of the previously described (Matselukh, 2010) mutant strain of *Bacillus* sp. 27, obtained with the help of N-methyl-N'-nitro-N-nitrosoguanidine, was 16.0 U/mg of protein. Since 1 mL usually contains about 1 mg of protein, the elastase activity of *Bacillus atrophaeus* 08 is much higher.

The fibrinogenolytic activity of *Bacillus licheniformis* 043 (41.66 U/mL) is almost 10 times higher than the activity of *Bacillus thuringiensis* IMV B-7324 (Nidialkova, 2014), with deep cultivation of which under certain conditions, an enzyme preparation with an activity of 4.17 U/mg of protein can be obtained. Today in Ukraine, there are no highly active producers of proteinases of microbial origin with fibrinogenolytic activity.

Currently, it is known (Suphatharaprateep & Jongjareonrak, 2011; Wanderley et al., 2017) that collagenases are synthesized by various microorganisms, many of which are pathogens for humans, which significantly limits the scope of their practical application. Therefore, non-pathogenic collagenase producers are of great theoretical and practical interest. Unfortunately, the collagenase activity of the *Bacillus subtilis* 248 strain we studied was insignificant (0.95 U/mL). It was several times lower than the activity of the collagenase enzyme preparation of *Streptomyces* sp. (Abdel-Fattah, 2013), as well as complex enzyme preparation *Bacillus thuringiensis* var. i*sraelensis* (Nidialkova & Chernyshenko, 2016), the collagenase activity of which reached 34.5 U/mg of protein, which is almost 13 times higher than the collagenase activity of *Streptomyces* sp. 1349. Therefore, *Bacillus subtilis* 248 as a collagenase producer is not promising for further research.

At the same time, *Bacillus subtilis* 248 and *Bacillus atrophaeus* 08, the elastase and fibrinogenolytic activities of which, respectively, are manifested at 12 °C on the 2nd day of their cultivation, may be promising for further research. Enzymes of psychrophilic microorganisms have attracted increased attention of researchers in recent years. This increased interest is attributed to the attractive properties of such proteins, namely high specific activity and low thermal stability, and thus, such cold-active enzymes represent a huge potential for basic research and biotechnological applications. Structural and functional studies of such enzymes allow for obtaining information on the mechanisms of their adaptation to functioning at low temperatures. Increasing the activity of enzymes obtained from cultures grown at low temperatures can be considered one of the adaptation mechanisms of the organism to environmental conditions. An important concept in adaptation to cold is the flexibility of the protein structure of such enzymes (Yang & Huang, 2023). The study of enzymes of marine microorganisms makes it possible to compare the properties of enzymes isolated from both thermophilic and psychrophilic microorganisms. This will make it possible to understand the molecular basis of cold or heat adaptation of enzymes of microorganisms that live in the appropriate conditions.

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О.В. Гудзенко[1], *Л.Д. Варбанець*[1], *В.О. Іваниця*[2]

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[2] Одеський національний університет ім. І.І. Мечникова,
вул. Дворянська, 2, Одеса, 65029, Україна

ВПЛИВ ТЕМПЕРАТУРИ КУЛЬТИВУВАННЯ НА ЕЛАСТАЗНУ, ФІБРИНОГЕНОЛІТИЧНУ ТА КОЛАГЕНАЗНУ АКТИВНОСТІ МОРСЬКИХ БАКТЕРІЙ *BACILLUS ATROPHAEUS 08, BACILLUS LICHENIFORMIS* 043 І *BACILLUS SUBTILIS* 248

Морські мікроорганізми є основними постачальниками життєво важливих органічних сполук у складній екосистемі Світового океану завдяки експресії цілого спектра унікальних ферментів, включаючи протеази. Активність протеаз залежить як від таксономічної приналежності штаму, так і від джерела та місця виділення мікроорганізму. Фактори довкілля бактерій, такі як солоність води, її температура, тиск, освітленість значною мірою визначають склад, фізико-хімічні властивості та субстратну специфічність продукованих ними ферментів. Оскільки не лише температура середовища мікроорганізмів, а й температура їх вирощування може істотно впливати на активність, **метою** роботи було визначити оптимальні температури культивування продуцентів, необхідні для досягнення максимальної еластазної, фібриногенолітичної та колагеназної активності досліджуваних штамів *Bacillus atrophaeus* 08, *Bacillus licheniformis* 043, *Bacillus subtilis* 248, які були виділені з різних глибин Чорного моря. **Методи**. Об'єктами дослідження були 3 штами: *Bacillus subtilis* 08, *Bacillus licheniformis* 043, *Bacillus subtilis* 248, виділені з донних відкладень із трьох точок на глибинах 888—2080 м у Чорному морі. Культури вирощували за температур 12, 28 і 42 °С зі швидкістю обертання 210 об/хв протягом 5 діб. Після закінчення ферментації біомасу відокремлювали центрифугуванням при 5000 g протягом 30 хв. Використовували методи визначення протеолітичної (еластолітичної, фібриногенолітичної та колагеназної) активності в супернатанті культуральної рідини. **Результати.** Хоча досліджувані культури були виділені майже з однакової глибини (*Bacillus atrophaeus* 08 і *Bacillus subtilis* 248 — 1499 м, *Bacillus licheniformis* 043 — 1537 м), супернатанти їхніх культуральних рідин проявляли різну ензиматичну активність в залежності як від температури, так і динаміки росту. Так, якщо для *Bacillus atrophaeus* 08 найбільша еластазна і колагеназна активності були виявлені за температури 28 °С на другу добу культивування, то фібриногенолітична – за 12 °С на другу добу вирощування. Максимальна еластазна і колагеназна активності *Bacillus licheniformis* 043 проявлялися за температури 28 °С на четверту і другу добу культивування відповідно**.** Найвища фібриногенолітична активність була за температури 42 °С на другу добу культивування. При вивченні впливу температури культивування *Bacillus subtilis* 248 показано, що максимальна еластазна активність досягається за температури 12 °С на другу добу культивування, найвища фібриногенолітична активність – при 28 °С на четверту добу культивування, а для досягнення максимальної колагеназної активності необхідно вирощувати культуру при 42 °С протягом чотирьох діб. **Висновки.** Температура культивування мікроорганізмів відіграє суттєву роль у досягненні максимальної протеолітичної актвності. Підбір оптимальної температури вирощування дозволяє збільшити в декілька разів еластазну, фібриногеназну і колагеназну активності. Встановлено, що динаміка синтезу у різних штамів суттєво відрізняється.

Ключові слова*: бактерії, виділені з глибоководних відкладень Чорного моря, температура культивування, еластолітична, фібриногенолітична, колагеназна активності.*

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Yu.B. PYSMENNA[1*], M.Ya. VORTMAN[2], V.N. LEMESHKO[2], V.V. SHEVCHENKO[2]

[1] Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine

[2] Institute of Macromolecular Chemistry, NAS of Ukraine,
48 Kharkivske Highway, Kyiv, 02160, Ukraine

* Author for correspondence; e-mail: yuliapysmenna@gmail.com

EFFECTIVENESS OF NEW ANTIFUNGAL DRUGS AGAINST MICROSCOPIC FUNGI ISOLATED FROM AGRICULTURAL CROPS

Guanidines are nitrogen-rich organic compounds that have been frequently associated with a wide range of biological activities, such as antibacterial, antiviral, antifungal, and antiprotozoal activities. Guanidinium-containing oligomers based on aliphatic and aromatic oligoepoxides are newly synthesized substances with antifungal activity, providing prospects for their use as agricultural fungicides. ***Aim.*** *The aim of the study was to investigate the effectiveness of new antifungal drugs against microscopic fungi isolated from agricultural crops and to determine the antagonistic activity of Trichoderma koningii and Trichoderma viride.* ***Methods.*** *The guanidine-containing alkyl-substituted oligomer was obtained through the reaction of the aromatic DER-331 or aliphatic DEG-1 oligoepoxide with guanidine, followed by interaction with alkyl bromides. The fungicidal activity was determined using the agar diffusion method on nutrient media with the following fungal strains: Alternaria alternata F-41618, Alternaria infectoria F-416121, Aspergillus niger F-41611, Aspergillus flavus F-41612, Acremonium strictum F-41615, Chaetomium globosum F-41617, Cladosporium sphaerospermum F-41623, Botrytis cinerea F-41603, Fusarium poae F-41610, and Fusarium moniliforme F-41605. To compare the effectiveness of the newly synthesized antifungal drugs with existing agents for treating agricultural crops, the following preparations were selected: «ROYALFLO», «MEDIAN EXTRA», and «STROBI». The determination of interspecies interactions among microscopic fungi was conducted using the agar block method with antagonist cultures isolated from soil, namely Trichoderma viride F-41256 and Trichoderma koningii F-41246.* ***Results.*** *The research results indicate that the newly synthesized fungicidal preparations from the group of guanidine-containing derivatives exhibit moderate fungicidal and fungistatic properties against these representatives of microscopic fungi. Our tests have demonstrated that the most effective biocide is a preparation of tetraalkyl-substituted oligomers based on an aromatic oligoepoxide. The strains of Alternaria infectoria, Aspergillus flavus, Acremonium strictum, Chaetomium globosum,*

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Cladosporium sphaerospermum, and Fusarium moniliforme showed high sensitivity to it, while Alternaria alternata exhibits — medium sensitivity. The strains of Aspergillus niger, Botrytis cinerea, and Fusarium poae displayed low sensitivity. The fungicide «STROBI» demonstrated the highest effectiveness among the biocidal preparations: the size of the growth retardation zone on the 14th day of cultivation decreased insignificantly compared to the other studied preparations. According to the experimental results, Trichoderma koningi exhibited superior antagonistic properties compared to Trichoderma viride. Acremonium strictum was nearly completely suppressed by the block culture Trichoderma viride. Significant fungicidal action with large zones of growth retardation was observed in the test cultures of Alternaria alternata and Cladosporium sphaerospermum. ***Conclusions.*** *It was found that newly synthesized guanidine-containing preparations exhibited moderate fungicidal and fungistatic properties. Although some drugs available on the market demonstrate much higher efficiency, the investigated compounds show promise due to their specific selectivity of action, especially in cases of resistance formation to other fungicidal drugs. Furthermore, the study demonstrates that the Trichoderma koningii strain exhibits a stronger antagonistic effect on fungi isolated from agricultural crops, offering the potential for the development of an effective antifungal agent.*

Keywords: *microscopic fungi, guanidine derivatives, antifungal drugs, antagonistic properties.*

Guanidine derivatives are widely utilized as biocides and disinfectants due to their broad spectrum of antimicrobial activity against both gram-positive and gram-negative bacteria, viruses, and fungi. The mechanism of the fungicidal action of polyguanidines is associated with the sorption of the biocidal polycation on the negatively charged cell membrane of microorganisms, leading to its destruction and cell lysis. In small concentrations, quaternary ammonium salts and polyguanidines disrupt membrane functions, causing alterations in osmotic pressure, permeability, and the rate of transfer of molecules and ions through the membrane. This disruption also inhibits metabolic processes and biological oxidation, leading to the inhibition of cell division (Brzezinska et al., 2018; Gomes et al., 2023; Lysytsya et al., 2023).

Fungal diseases are a significant cause of crop yield losses and a decline in product quality. Consequently, the search for new antifungal drugs is a highly urgent task. Filamentous fungi are responsible for many postharvest losses of fruits and vegetables, posing a substantial challenge to quality preservation, particularly after harvesting. Fungal activity can lead to contamination with mycotoxins, posing potential health risks to consumers (Peng et al., 2021).

Currently, the market offers a wide range of fungicidal preparations, both for treating crops and preserving finished products for long-term storage. With a strong biocidal effect against many microorganisms, polyguanidines are considered safe for humans and animals. They belong to the IV class of substances slightly harmful when in contact with the skin and the III class of moderately harmful compounds when ingested. Aqueous solutions of polyguanidines are stable, retaining biocidal activity for an extended period. They have no odor, do not cause discoloration of fabrics, and do not corrode equipment. Additionally, they exhibit surface-active properties (Koffi-Nevryet al., 2011; Peng et al., 2021).

However, the prolonged use of the same fungicides leads to the development of resistance in fungi against their action. Therefore, the task of finding new drugs, including those based on natural antagonists of phytopathogenic fungi, is urgent. It is known from the literature that species of the genus *Trichoderma* can not only inhibit the growth of phytopathogens of the genus *Fusarium* but also use the pathogen as a substrate (hyperparasitism). Moreover, the development of *Trichoderma* fungi is not harmful to human health, making them promising candidates for the creation of biological preparations. (Yassin et al., 2021).

The **aim** of this work was to investigate the effectiveness of new antifungal drugs against microscopic fungi isolated from agricultural crops

and to determine the antagonistic activity of *Trichoderma koningii* and *Trichoderma viride.*

Materials and Methods. The following types of microscopic fungi were used as objects of research to determine the fungicidal activity of biocidal preparations and their antagonistic activity (Table 1).

The fungicidal activity was determined using the agar diffusion method on nutrient media (Vortman et al., 2020). The sensitivity of fungi isolated from crops to the investigated compounds was assessed by measuring the diameter of the zone of growth retardation of micromycetes: > 25 mm — high; 25—15 mm — moderate; < 15 mm — low; 0 mm — absent. To compare the effectiveness of newly synthesized antifungal drugs with existing agents for treating crops, the following preparations were selected: «ROYAL-FLO», «MEDIAN EXTRA,» and «STROBI».

The determination of interspecies interactions of microscopic fungi was carried out using the agar block method (Pysmenna et al., 2016), which allows us to reveal the nature of the interaction between the test culture and the block culture.

The experiment was conducted in three repetitions over 14 days. Interactions among species of microscopic fungi were assessed based on a set of criteria, including the diameter of the zone of growth retardation, its change during the study, and comparison with the development of control blocks. This assessment followed a system with the following categories (Pysmenna et al., 2016): I — inhibition by the culture block, II — fungicidality of the block, III — fungistatic action of the culture block, IV — interaction of the culture-block with absent or weakly expressed antagonistic properties, V — fungistatic action of the test culture, and VI — neutral interaction of two cultures which develop evenly.

Table 1. **Types of microscopic fungi used for research**

N	Type of microscopic fungus	Strain number	Source of isolation
	Micromycetes isolated from agricultural crops and granary premises		
1	*Alternaria alternata*	F-41618	Tomato
2	*Alternaria infectoria*	F-416121	Tomato
3	*Aspergillus niger*	F-41611	Granary premises
4	*Aspergillus flavus*	F-41612	Wheat
5	*Acremonium strictum*	F-41615	Granary premises
6	*Chaetomium globosum*	F-41617	Wheat
7	*Cladosporium sphaerospermum*	F-41623	Tomato
8	*Botrytis cinerea*	F-41603	Grape
9	*Fusarium poae*	F-41610	Wheat
10	*Fusarium moniliforme*	F-41605	Wheat
	Antagonist cultures		
11	*Trichoderma viride*	F-41256	Soil
12	*Trichoderma koningii*	F-41246	Soil

Synthesis of oligomers. Diane epoxy oligomer DER-331 (DOW Chemical Company, Germany), MM 365 g/mol, mass fraction of epoxy groups 23.5%, hydroxyl groups 0.6%, and aliphatic epoxy oligomer DEG-1 (ToV «Spetskontrakt»), mass fraction 28.6% of epoxy groups, 1.3% of hydroxyl groups, were dehydrated by heating in a vacuum for 2—6 hours at 80—90 °C and a final pressure of 2 mm Hg. Guanidine hydrochloride (GD) (Aldrich, 99.9% purity) was used without additional purification. Alkyl bromides, namely heptyl-(C_7H_{15}) and decyl-($C_{10}H_{21}$) bromides (Aldrich, 99.9% purity), were used without additional purification. Methanol was purified by distillation. ChDA brand dimethylformamide was used without further purification.

The synthesis of tetraalkyl-substituted guanidinium-containing oligomers was conducted in two stages. At the first stage, guanidine-containing oligomers with terminal guanidine fragments were obtained by reacting guanidine, previously converted with the help of alkali from the salt form to the base, with aromatic DER-331 or aliphatic DEG-1 oligoepoxide in dimethylformamide at a temperature of 70°C for 4 hours, with a molar ratio of components of 2:1 (Feiertag et al., 2003; Palátet al., 2007).

At the second stage, the reaction between guanidinium-containing oligomers and alkyl bromides (Alk= -C_3H_7,- C_7H1_5, $C_{10}H_{21}$) was carried out in methanol at 50 ^{0}C for 2—3 hours and a molar ratio of components of 1:4. The obtained products were reprecipitated from methanol into diethyl ether. To remove the solvent residues, the product was kept in a vacuum at a 60 °C for 5 hours. Product yield was 93—95%. Therefore, the following tetraalkyl-substituted guanidine-containing oligomers were obtained:

$^{-}$Br Alk, $^{-}$Br Alk N^{+} – C(=NH) – NH – CH_2–CH(OH)–CH_2 – R – CH_2–CH(OH)–CH_2 – NH – C(=NH) – N^{+} Alk $^{-}$Br, Alk $^{-}$Br

Alk = C_7H_{15}, $C_{10}H_{21}$

- based on aromatic oligoepoxide

R = [–O–C_6H_4–C–C_6H_4–O–CH_2–CH(OH)–CH_2–]$_{0.07}$–O–C_6H_4–C–C_6H_4–O–

- based on aliphatic oligoepoxide

R = CH_2CH_2 O CH_2CH_2

Results. The antifungal properties of three biocidal preparations belonging to the group of guanidine-containing derivatives synthesized by the Institute of High Technologies (Ukraine) and preparations «ROYAFLO», «MEDIAN EXTRA», and «STROBI» were studied.

The scheme for obtaining alkyl-substituted guanidinium oligomers can be presented as follows:

(epoxide)–R–(epoxide) + 2 H_2N–C(=NH)–NH_2 →

→ H_2N–C(=NH)–NH–CH_2–CH(OH)–CH_2–R–CH_2–CH(OH)–CH_2–NH–C(=NH)–NH_2 + 4 Alk Br^{-} →

→ $^{-}$Br Alk, $^{-}$Br Alk N^{+}–C(=NH)–NH–CH_2–CH(OH)–CH_2–R–CH_2–CH(OH)–CH_2–NH–C(=NH)–N^{+} Alk $^{-}$Br, Alk $^{-}$Br

R = [–O–C_6H_4–C–C_6H_4–O–CH_2–CH(OH)–CH_2–]$_{0.07}$–O–C_6H_4–C–C_6H_4–O–

R = $CH_2CH_2OCH_2Ch_2$ Alk = C_7H_{15}, $C_{10}H_{21}$

Control over the completion of the reaction was carried out by IR spectroscopy. The IR spectra bands: 3200—3550 cm^{-1} and bands of valence vibrations of OH and NH groups; 2869 cm^{-1}, 2926 cm^{-1}, and 2964 cm^{-1} — absorption bands of -CH, -CH2, and -CH3 groups, 1460 cm^{-1} — deformation vibrations of the C—H bond, 1640 cm_{-1} — valence vibrations of C=N guanidine fragments and deformation vibrations of NH groups. 1450—1650 cm^{-1} — absorption bands of C=C bonds of the benzene ring for an oligomer based on an aromatic oligoepoxide, 1640 cm^{-1} — deformation vibrations of NH groups, and 1100—1300 cm^{-1} — vibrations of C—O—C bonds of ether groups (Fig. 1).

The structure of the obtained oligomers was confirmed by the method of ^{1}H-NMR spectrometry. In the ^{1}H NMR ($CDCI_3$) spectrum of tetraalkyl-substituted guanidinium-containing oligomers, proton signals are present at 1.72 ppm (t, 3H, -CH_3), 2.73 ppm -NH (NH CH_2), 2.58 ppm -CH_2 (CH_2CHOH), 3.58 ppm -OH (CH-OH)), 3.96 ppm -CH (CH-OH), 6.8 ppm and 7.2 ppm -CH of the benzene ring for the oligomer based on the aromatic oligoepoxide, and 7.8 ppm and 8.2 ppm -NH (NH_{20}) groups (Fig. 2).

The effect of fungicides on the studied micromycetes is shown in Fig. 3

According to the obtained results, it can be concluded that the fungicide «STROBI» exhibits the greatest effectiveness among the biocidal preparations studied. The size of the zone of growth retardation on the 14th day of cultivation decreased insignificantly compared to the other preparations. The lowest efficiency was observed in the following species: *Aspergillus niger, Botrytis cinerea,* and *Fusarium poae*; on the other hand, it caused complete inhibition of growth in other species.

The composition of the fungicide «STROBI» includes the active substance of the class of strobilurins — kresoxim-methyl, which is a fungicide with protective, curative, eradication, and long-term residual control of the disease. The mechanism of its action consists in inhibiting the

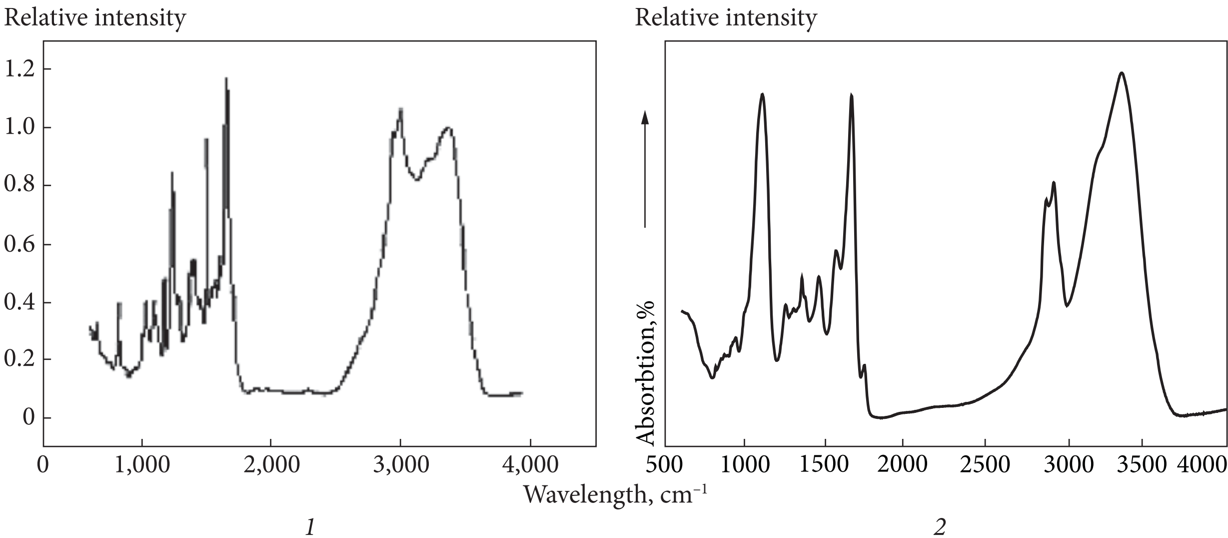


Fig. 1. IR spectra of tetraalkyl-substituted oligomers: *1* — based on aromatic oligoepoxide (Alk= $C_{10}H_{21}$), *2* — based on aliphatic oligoepoxide (Alk= C_7H_{15})

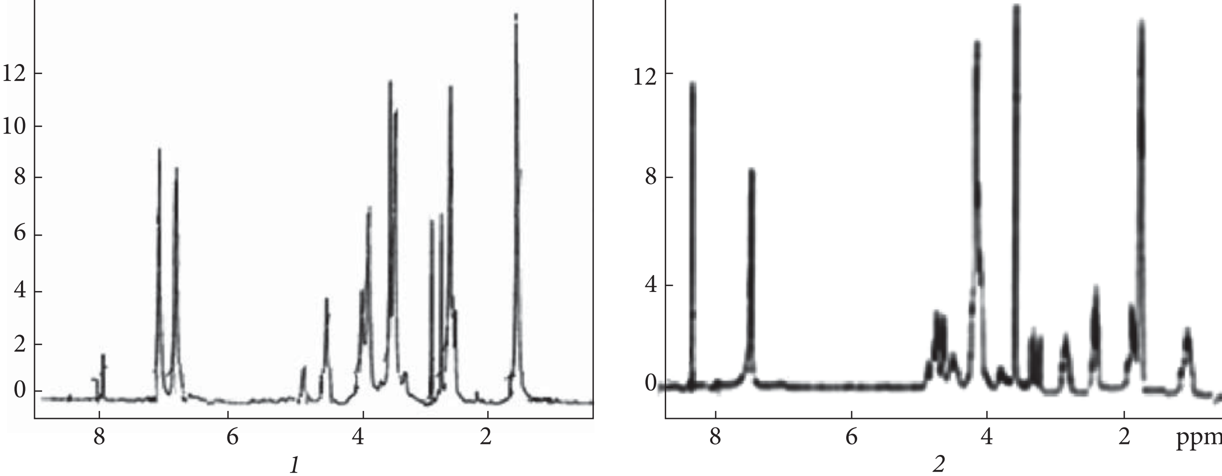


Fig. 2. ^{1}H NMR spectra of tetraalkyl-substituted oligomers: *1* — based on aromatic oligoepoxide (Alk= $C_{10}H_{21}$), *2* — based on aliphatic oligoepoxide (Alk= C_7H_{15})

germination of spores due to blocking the transport of electrons in the mitochondria of cells, thus preventing the formation of ATP, necessary for the normal metabolic process of the microscopic fungi. Kresoxim-methyl has low toxicity for mammals, invertebrates, and arthropods and medium toxicity for birds. It is classified as very toxic to aquatic organisms (fish, algae, and aquatic invertebrates).

The second most effective fungicide is «ROYALFLO», the maximum effectiveness of which can be observed for the following species: *Alternaria infectoria, Aspergillus flavus, Acremonium strictum, Chaetomium globosum,* and *Cladosporium sphaerospermum,* moderate effectiveness — for *Alternaria alternata* and *Botrytis cinerea*, and poor one — for *Aspergillus niger, Fusarium poae,* and *Fusarium moniliforme.* It

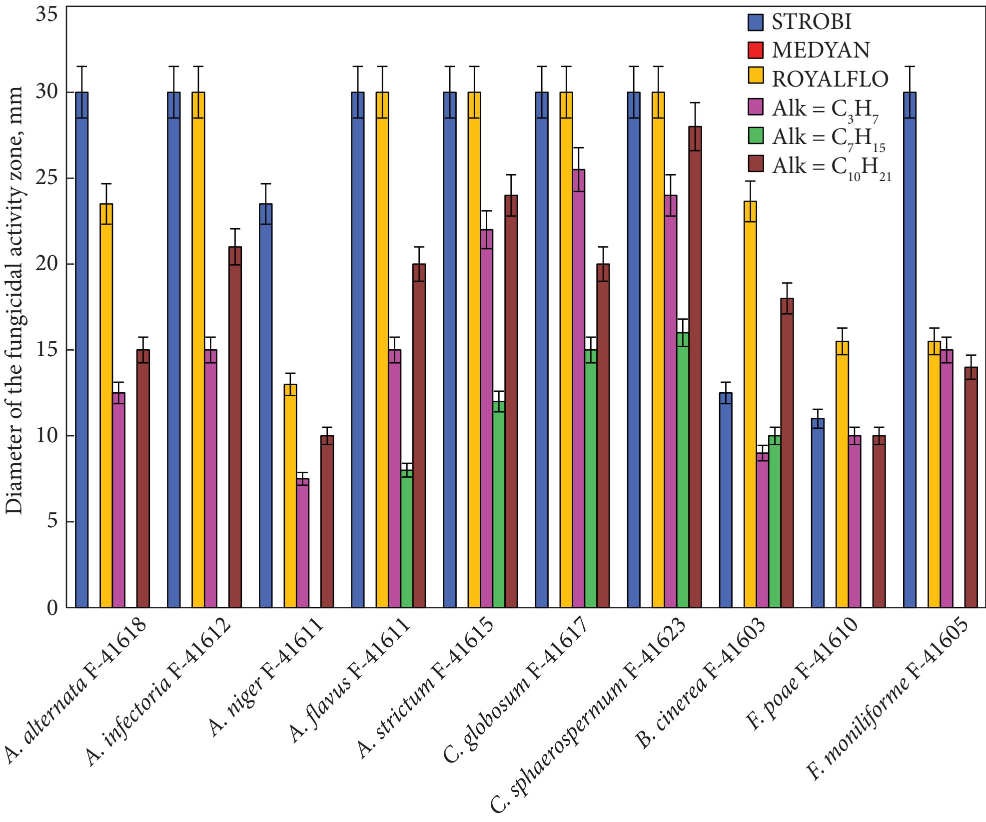


Fig. 3. Effectiveness of anitifungal drugs against micromycetes-contaminants of agricultural crops

should be also noted a slight correlation in the sizes of growth retardation zones on the 7th and 14th day of cultivation. «ROYALFLO» is a contact, highly effective fungicide, whose main active ingredient is thiram.

The drug «MEDIAN EXTRA» showed the least effectiveness against the studied species of micromycetes, since the use of the drug did not delay the process of culture development on some studied cups, and even the development of symbiotic representatives was observed. This fungicide showed low effectiveness against the representative of *Fusarium moniliforme*: an insignificant zone of growth retardation was observed only on the 7th day of cultivation, and on the 14th day, the drug did not show antifungal properties (Figs. 3 and 4).

«MEDIAN EXTRA» is a broad-spectrum contact fungicide with copper as an active substance. Due to its strong affinity for amino acids and carboxyl groups, copper reacts with proteins and acts as an inhibitor of enzymes in target organisms.

The research results indicate that the newly synthesized fungicidal preparations from the group of guanidine-containing derivatives exhibit moderate fungicidal and fungistatic properties toward these representatives of microscopic fungi (Fig. 5).

Our tests showed that the most effective biocide is a preparation of tetraalkyl-substituted oligomers based on an aromatic oligoepoxide, the sensitivity to which of the strains *Alternaria infectoria, Aspergillus flavus, Acremonium strictum, Chaetomium globosum, Cladosporium sphaerospermum, and Fusarium moniliforme* is high, *Alternaria alternata* — medium, and *Aspergillus* spp. *niger*, *Botrytis cinerea,* and *Fusarium poae* — low. This fungicide was most effective against the

species *Cladosporium sphaerospermum* and the lowest against *Botrytis cinerea* (Figs. 3 and 5). It is important to note that this drug worked against all types of microscopic fungi tested.

In general, the drug Alk=C_3H_7 showed worse fungicidal and fungistatic qualities compared to the previous one. *Alternaria infectoria, Aspergillus flavus, Acremonium strictum, Chaetomium globosum,* and *Cladosporium sphaerospermum* showed high sensitivity to it; *Botrytis cinerea* and *Fusarium moniliforme* — medium sensitivity, and *Alternaria alternata, Aspergillus niger*, and *Fusarium poae* — low sensitivity. Among fungicides derived from guanidine, this drug proved to be the most effective toward test cultures of *Alternaria infectoria, Aspergillus flavus,* and *Cladosporium sphaerospermum*.

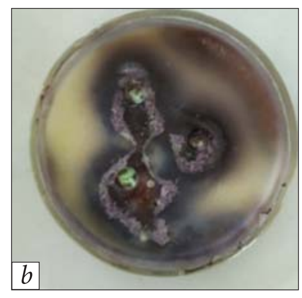
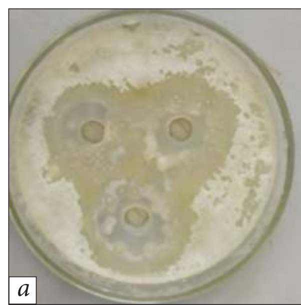
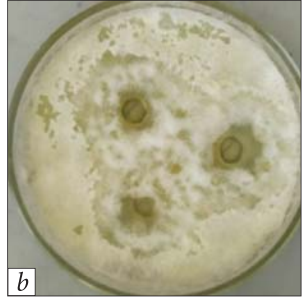


Fig. 4. Effectiveness of the drug «MEDIAN EXTRA» against the representative of *Fusarium moniliforme*: *a* — zone of growth retardation on the 7th day of cultivation, *b* — the complete absence of action on the 14th day of cultivation

Biocidal drug Alk=C_7H_{15} was not sensitive to test cultures *Alternaria alternata, Alternaria infectoria, Aspergillus niger, Aspergillus flavus, Fusarium poae,* and *Fusarium moniliforme*. As for *Aspergillus flavus*, its sensitivity was lower compared to *Acremonium strictum, Chaetomium globosum, Cladosporium sphaerospermum*, and *Botrytis cinerea* in the growth retardation zone up to 25 mm, so the sensitivity (as a whole) to the drug was average.

To determine the effectiveness of using *Trichoderma koningii* and *Trichoderma viride* cultures as biological preparations to reduce the number of phytopathogenic fungi, their antagonistic properties were assessed. According to the results obtained on the Chapek-Dox medium, interaction category V was most frequently detected — the lawn culture exhibited a fungistatic effect on the antagonist culture. Interaction categories II — fungicidal effect of the block, and VI — neutral interaction of two cultures — occurred with the same frequency. The categories of interactions between test crop lawns and antagonist blocks are listed in Table 2.

The antagonistic activity of the *Trichoderma viride* test culture, according to our data, demonstrated a fungicidal effect on *Acremonium strictum* and *Cladosporium sphaerospermum*, and a fungistatic effect on *Alternaria alternata*. In general, *Trichoderma viride* was fungistatically affected by most of the test cultures, including

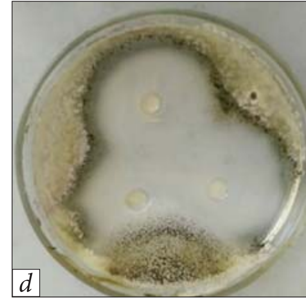


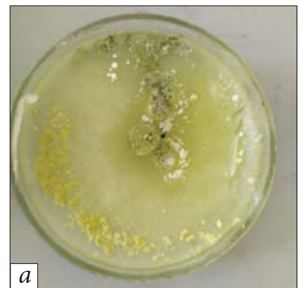
Fig. 5. Representatives with the lowest and highest sensitivity to the fungicide Alk= $C_{10}H_{21}$: A — growth retardation zones on the 7th day of cultivation in the *Botrytis cinerea* strain, B — growth retardation zones on the 14th day of cultivation in the *Botrytis cinerea* strain, C — zones of growth retardation on the 7th day of cultivation in the *Botrytis cinerea* strain, D — zones of growth retardation on the 14th day of cultivation in the *Cladosporium sphaerospermum* strain

Alternaria infectoria, Aspergillus niger, Chaetomium globosum, Botrytis cinerea, Fusarium poae, and *Fusarium moniliforme*. A neutral interaction between the two cultures was observed only with *Aspergillus flavus* (Fig. 6).

According to the results of the experiment, *Trichoderma koningi* has better antagonistic properties compared to *Trichoderma viride*. *Acremonium strictum* was almost completely suppressed by the block culture. Bright fungicidal action with large zones of growth retardation was observed on test cultures *of Alternaria alternata* and *Cladosporium sphaerospermum*. Weakly expressed antagonistic properties were observed against the *Botrytis cinerea* species. Uniformly and independently, lawn and block developed with test cultures of *Aspergillus flavus* and *Chaetomium globosum*. *Alternaria infectoria, Aspergillus niger, Fusarium poae,* and *Fusarium moniliforme* completely blocked the development of the block culture (Fig. 7).

Discussion. Our tests showed that the most effective biocide is a preparation of tetraalkyl-substituted oligomers based on an aromatic oligoepoxide. In comparison with our earlier studies, it was established that alkyl-substituted (Alk=$C_7H_{15}Br$, $C_{10}H_{21}Br$) guanidinium-containing oligomers at a concentration of 1% exhibited fungicidal activity against almost all tested isolates. Conversely, the oligomer with a heptyl radical did not show fungicidal activity at all, and this pattern was confirmed in the conducted experiment. It is important to note that the zones of growth retardation for isolates such as *A. flavus* and *A. niger* were 26.6 mm and 14.6 mm, respectively, which significantly exceed the previously obtained indicators (Vortman et al., 2020).

The obtained data indicate the selectivity of the fungicidal action of the solutions on different types of microscopic fungi, which may be associated with differences in their metabolic processes and adaptation mechanisms. According to the results of microbiological studies, alkyl-substituted guanidinium-containing oligomers show



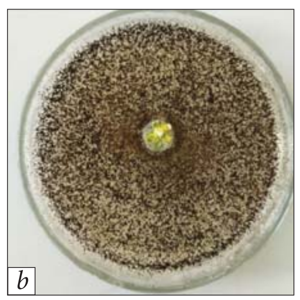
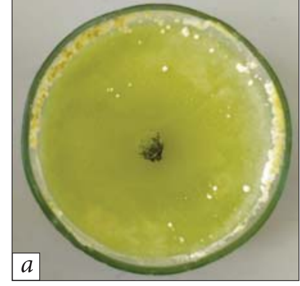


Fig. 6. Categories of interaction of *Trichoderma viride* with cultures of the block: A — II — fungicidal effect of the block on *Acremonium strictum*; B — V — fungistatic action of *Aspergillus niger*



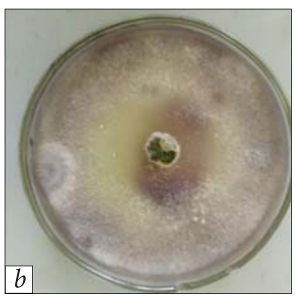


Fig. 7. Categories of the interaction of *Trichoderma koningii* with block cultures: A — I — suppression of *Acremonium strictum* by the block culture; B — V — fungistatic effect of *Fusarium moniliforme*

Table 2. **Interspecies interaction of antagonistic fungi and test cultures**

Test culture	*Trichoderma viride*	*Trichoderma koningii*
Alternaria alternata	III	II
Alternaria infectoria	V	V
Aspergillus niger	V	V
Aspergillus flavus	VI	VI
Acremonium strictum	II	I
Chaetomium globosum	V	VI
Cladosporium sphaerospermum	II	II
Botrytis cinerea	V	IV
Fusarium poae	V	V
Fusarium moniliforme	V	V

I — oppression by the block culture of the lawn; II — fungicidal action of the block; III — fungistatic action of the block culture k; IV — culture-block interaction with absent or weakly expressed antagonistic properties; V — fungistatic action of the lawn culture on the block culture; VI — neutral interaction of two cultures.

fungicidal activity against microscopic fungi and antimicrobial activity against the test cultures, recommended for commercial disinfectants. By the literature, the mechanism of fungicidal action of guanidine polymers is associated with adsorption on the negatively charged cell surface and blocking the transport of metabolites through the cell wall and cytoplasmic membrane. It can be assumed that the compounds studied by us exhibit antifungal activity by a similar mechanism. It is worth noting the low toxicity of such compounds for humans and warm-blooded animals (Peng et al., 2021; Gomes et al., 2023).

By our data, micromycetes *Trichoderma koningii* and *Trichoderma viride* have a fairly high antagonistic activity (fungicidal and fungistatic effects) against micromycetes isolated from crops, that is, they are promising species for creating biological preparations based on them.

Several antagonistic mechanisms are used against plant pathogens. These include antibiosis, mycoparasitism, competition for nutrients and space, plant growth stimulation, inducible plant defense mechanisms, and modification of environmental conditions. Mycoparasitism is a direct mechanism of biological control that works by parasitizing, detecting, growing, and colonizing the pathogen. The ability to mycoparasitize other fungi is widely used for biological control of agricultural pests (mainly against pathogenic fungi and parasitic nematodes) (Peng et al., 2021; Yassin et al., 2021).

Antimicrobial activity may result from several secondary metabolites such as terpenes, polyketides, gliotoxin, and gliovirin produced by fungi. Other metabolites include tricholine, garzianic acid, viridian, gliosoprinins, heptelic acid, 6-pentyl-α-pyrone, and massoylactone.

The obtained data indicate that micromycetes of the genus *Trichoderma* spp. are potentially effective biological control agents and can be used as active ingredients in biopesticides, biofertilizers, growth enhancers, and natural resistance promoters. This is due to their ability to protect plants, enhance vegetative growth, and maintain pathogen populations in numerous agricultural conditions, as well as act as soil inoculant additives to improve nutrient properties, decomposition, and biodegradation (Al-Ani, 2018; Pysmenna et al., 2016).

Conclusions. It was found that newly synthesized guanidine-containing preparations exhibit average fungicidal and fungistatic properties against microscopic fungi isolated from agricultural plants. Although some drugs available on the market demonstrate much higher efficiency, the investigated compounds are promising for use due to their specific selectivity of action, especially in cases of the formation of resistance to fungicidal drugs.

When investigating the potential use of *Trichoderma koningii* and *Trichoderma viride* strains for the production of biological preparations, it was determined that *Trichoderma koningii* had better antagonistic properties compared to *Trichoderma viride*. *Acremonium strictum, Alternaria alternata*, and *Cladosporium sphaerospermum* cultures had the largest zones of growth retardation.

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Ю.Б. Письменна[1], М.Я. Вортман[2], В.Н. Лемешко[2], В.В. Шевченко[2]*

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[2] Інститут хімії високомолекулярних сполук НАН України,
Харківське шосе, 48, Київ, 02160, Україна

ЕФЕКТИВНІСТЬ НОВИХ АНТИФУНГАЛЬНИХ ПРЕПАРАТІВ ЩОДО МІКРОСКОПІЧНИХ ГРИБІВ, ВИДІЛЕНИХ З СІЛЬСЬКОГОСПОДАРСЬКИХ КУЛЬТУР

Гуанідини — азотисті органічні сполуки, які мають різноманітні біологічні активності, такі як антибактеріальна, антивірусна, антигрибкова та антипротозойна дія. Олігомери на основі гуанідину, що містять аліфатичні та ароматичні олігоепоксиди, є новосинтезованими речовинами з антифунгальною активністю, що відкриває перспективи їх використання як фунгіцидних засобів для сільськогосподарських культур. **Метою** дослідження було вивчити ефективність нових антифунгальних препаратів проти мікроскопічних грибів, виділених із сільськогосподарських культур та визначити антагоністичну дію *Trichoderma koningii* і *Trichoderma viride*. **Методи.** Гуанідинвмісний алкільно-заміщений олігомер було отримано реакцією ароматичного DER-331 або аліфатичного DEG-1 олігоепоксиду з гуанідином, за якою відбувалася взаємодія з алкільними бромідами. Антифунгальну активність визначали методом дифузії в агар на поживних середовищах зі штамами грибів: *Alternaria alternata* F-41618, *Alternaria infectoria* F-416121, *Aspergillus niger* F-41611, *Aspergillus flavus* F-41612, *Acremonium strictum* F-41615, *Chaetomium globosum* F-41617, *Cladosporium sphaerospermum* F-41623, *Botrytis cinerea* F-41603, *Fusarium poae* F-41610, *Fusarium moniliforme* F-41605. Для порівняння ефективності новосинтезованих препаратів із наявними засобами для обробки сільськогосподарських культур обрано препарати «ROYALFLO», «МЕДЯН ЕКСТРА» та «СТРОБІ». Визначення міжвидових взаємодій між мікроскопічними грибами проводилося методом агарових блоків з антагоністичними культурами, ізольованими з ґрунту — *Trichoderma viride* F-41256, *Trichoderma koningii* F-41246. **Результати.** Результати дослідження показали, що новосинтезовані фунгіцидні гуанідинвмісні препарати показали середню фунгіцидну та фунгістатичну дію на мікроскопічні гриби. Показано, що найефективніший біоцид — препарат на основі тетраалкіл-заміщених олігомерів на основі ароматичного олігоепоксиду. Висока чутливість спостерігається у штамів *Alternaria infectoria*, *Aspergillus flavus*, *Acremonium strictum*, *Chaetomium globosum*, *Cladosporium sphaerospermum*, *Fusarium moniliforme*, тоді як *Alternaria alternata* має середню чутливість. Штами *Aspergillus niger, Botrytis cinerea* та *Fusarium poae* проявляють низьку чутливість. Фунгіцид «STROBI» продемонстрував найвищу ефективність серед біоцидних препаратів. Розмір зони затримки росту на 14-й день культивування зменшився незначно у порівнянні з іншими вивченими препаратами. Виявлено, що *Trichoderma koningi* має більш виражені антагоністичні властивості порівняно з *Trichoderma viride*. Ріст *Acremonium strictum* практично повністю був пригнічений культурою блоку *Trichoderma viride*, фунгіцидна дія з великими зонами затримки росту спостерігалась на тестових культурах *Alternaria alternata* та *Cladosporium sphaerospermum*. **Висновки.** Новосинтезовані гуанідинвмісні препарати виявили середню фунгіцидну та фунгістатичну дію. Незважаючи на те, що деякі препарати, які доступні на ринку, демонструють набагато вищу ефективність, вивчені сполуки є перспективними з огляду на селективність їхньої дії, особливо в разі формування стійкості до дії фунгіцидних препаратів. Крім того, дослідження показало, що штам *Trichoderma koningii* виявляє сильнішу антагоністичну дію на гриби, ізольовані із сільськогосподарських культур, що відкриває можливості для розробки ефективного антифунгального засобу.

Ключові слова: *мікроскопічні гриби, гуанідінові похідні, антифунгальні препарати, антагоністичні властивості.*

SHORT COMMUNICATIONS

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A.V. PLUZHNYK[1,2*], V.R. PETLOVANA[1], V.V. DZHAGAN[1]

[1] Taras Shevchenko National University of Kyiv,
64/13 Volodymyrska Str., Kyiv, 01601, Ukraine

[2] Kholodnyi Yar National Nature Park,
58 Kholodnoyarska Str., Hruskivka, Cherkaska oblast, 20810, Ukraine

[*] Author for correspondence; e-mail: andriy.pluzhnik@knu.ua

RAPID AND EFFICIENT METHOD FOR DNA EXTRACTION FROM FUNGI OF THE GENUS *MORCHELLA* DILL. EX PERS.

The taxonomic features of true morels (genus Morchella, Ascomycota) have long been a difficult problem because the identification of species of such a taxonomically complex genus is impossible without the use of molecular phylogenetics methods. To date, there are a large number of DNA extraction methods, but the search for a fast and efficient method of DNA extraction from various organisms, including fungi, remains an urgent problem in modern biological research. ***Methods.*** *The study material consisted of the fruiting bodies of fungi belonging to the genus Morchella. Total DNA extraction was performed using the original DNA Microprep Isolation from Plants protocol (DNA Microprep method). For comparison, the modified CTAB method was also used to extract DNA from all samples. Extraction efficiency was determined by spectrophotometry. The ITS marker sequence of the ribosomal RNA nuclear gene cluster was amplified using primers ITS1 and ITS4, and the amplification products were separated by electrophoresis in an agarose gel.* ***Results.*** *DNA-containing pellets were formed in all samples extracted by the DNA Microprep method, as confirmed by spectrophotometric analysis. The extracted nucleic acid was of acceptable quantity and purity. The amplification of the ITS marker sequence was successful for all samples isolated by the DNA Microprep method, resulting in amplicons of different lengths, which may indicate a potential difference in the taxonomic affiliation of the studied fruiting bodies of morels. Regarding PCR products based on DNA extracted by the CTAB method, 80% of the samples tested were positive for PCR amplification.* ***Conclusions.*** *The study suggests that the DNA Microprep method is a convenient and effective way to extract DNA from morels' fruit bodies. The method offers advantages such as a short extraction process, availability of reagents, and environmental friendliness. Additionally, PCR products were successfully obtained from the extracted DNA. To accurately identify the species of the taxonomically complex genus Morchella, it is necessary to determine the nucleotide sequences of the obtained amplicons and perform a phylogenetic analysis.*

Keywords: *DNA extraction, fungi, Morchella, DNA amplification, PCR.*

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The taxonomic classification of the *Morchella* genus has been a subject of controversy due to the difficulty in morphologically distinguishing true morels. This is because of their phenotypic plasticity and lack of taxonomically useful characters (Kuo et al., 2012). Hawksworth et al. recognize 28 species within the genus *Morchella* (Hawksworth, 1995), while Kirk et al. note 36 species (Kirk et al., 2008). Some taxonomists cite up to 50 species, while others may be limited to 3—6 species (Weber & Weber, 1988). Currently, the genus *Morchella* has about 80 verified species, but the actual number could be closer to 100, considering the significant number of recently described new species (Clowez et al., 2022), as well as unpublished species in herbaria. Thus, there is considerable confusion in the literature regarding the number of authentic morel species. However, identifying species of such a taxonomically complex genus is currently impossible without molecular studies that utilize various molecular markers (the large subunit of the nuclear ribosomal RNA (LSU), ribosomal small subunit (SSU), the nuclear ribosomal internal transcribed spacer (ITS), RNA polymerase II largest subunit (RPB1) along with the RNA polymerase II second largest subunit (RPB2), and the translation elongation factor 1-alpha (EF1-α)) and implement molecular phylogenetic methods (Loizides et al., 2022; Sa et al., 2022; Cravero et al., 2023; Li et al., 2024).

The first step in any molecular phylogenetic study is DNA extraction. Therefore, a crucial aspect of modern molecular biological research on fungi is to identify the most efficient method of DNA extraction. This method should produce a high-quality product in a short time and with minimal use of low-toxic reagents.

Currently, there are a large number of methods for extracting DNA from fungi, which produce different yields of fungal DNA (Fredricks et al., 2005; Dörnte & Kües, 2013; Aamir et al., 2015; Mykchaylova et al., 2017; Clowez et al., 2022). There are also many commercially available DNA extraction kits (Sa et al., 2022; Snabl et al., 2023; Wani et al., 2023; Li et al., 2024), but their use is often limited by high cost.

The extraction of genomic DNA is usually done with cetyltrimethylammonium bromide (CTAB) extraction buffer followed by purification through phenol/chloroform extraction and precipitation with isopropanol or ethanol (Gardes & Bruns, 1993). The only limitations of this method may be the harmful nature of some solvents and the rather long isolation procedure. Currently, this method with various modifications is quite popular (Liu et al., 2014; Huang et al., 2018; Petrželová & Sochor, 2019).

Furthermore, common methods require sufficient amounts of fungal material, are time-consuming due to extensive handling and purification steps, involve costs for chemicals, and are an extra investment when applied in the form of commercial kits.

The DNA MicroPrep Isolation from Plants method (Dellaporta, 1994) is widely used for DNA extraction from various plants. However, there is no literature available on its effectiveness for extracting DNA from fungi.

The study **aimed** to evaluate the efficiency of the DNA MicroPrep Isolation from Plants method (DNA Microprep) for extracting DNA from fruiting bodies of *Morchella* and to analyze its suitability for species identification.

Materials and Methods. The sample material consisted of the fruiting bodies (ascocarps) collected during the spring season of 2023 from the western outskirts of the Kholodnyi Yar National Nature Park (Cherkaska oblast), as well as from household plot in Fastiv (Kyivska oblast) (Table 1). Macromorphological characteristics of the morels' ascocarps were recorded during field research, including information on their color, size, shape, and texture.

DNA was extracted from fresh and dried fruiting bodies using two methods — the DNA MicroPrep Isolation from Plants and CTAB method.

According to the DNA Microprep protocol, to homogenize the material, small samples of fruiting bodies (3—5 mm^3) were placed in microtubes and immersed in liquid nitrogen. Instead of using a mechanized homogenizer, a small amount of carborundum was added to the pieces of fungal fruiting bodies and crushed with a pestle. Next, 750 μL of extraction buffer (1% SDS, 100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 10 mM 2-mercaptoethanol (added immediately before use)) was added to each tube and mixed thoroughly. The tubes were incubated at 65 °C for 10 min.

Following the initial step, 200 μL of 3M sodium acetate solution was added to the samples and mixed thoroughly. The mixture was incubated on ice for 20 minutes. All further manipulations were carried out at room temperature. Subsequently, the material was centrifuged at maximum speed (18,000 g in our case) for 10 minutes. The supernatant was then transferred to new tubes and an equal volume of isopropanol was added and mixed thoroughly. Finally, the resulting mixture was centrifuged at maximum speed for 2 minutes. The supernatant was then removed, and 80% ethanol solution (450 μL) was added to the resulting DNA pellet. The mixture was centrifuged again at maximum speed for 2 minutes, after which the ethanol was poured off, and the tubes were dried until the ethanol evaporated completely. Finally, the resulting DNA was dissolved in a 50 μL TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

For comparison, the modified CTAB method, as described in the appropriate protocol (Tarieiev et al., 2011), was used to extract DNA from all samples. The DNA extraction was assessed visually by the formation of pellets at the final stage of extraction. The extraction efficiency was assessed using both electrophoresis and spectrophotometry with a DS-11 FX+ spectrophotometer (DeNovix, USA).

The amplification of the ITS marker sequence of the nuclear ribosomal RNA gene cluster was performed using primers ITS1(TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). The expected size of the amplification product is approximately 750 base pairs. PCR was carried out using MyTaq Reaction Buffer Colorless (Meridian Bioscience Inc., USA) and Pfu-X Polymerase.

Table 1. **Information about the fruiting bodies of *Morchella* spp. used for DNA extraction**

No.	Photo of the sample	Collection number and type of the sample	Place of finding
1		KY1872 (dried)	Kholodnyi Yar National Nature Park, oak and hornbeam forest edge
2		KY0901 (fresh)	Kholodnyi Yar National Nature Park, steppe area
3		KY0881 (fresh)	Kholodnyi Yar National Nature Park, steppe area
4		FS0082 (dried)	a private plot, Fastiv
5		KY1999 (fresh)	Kholodnyi Yar National Nature Park, oak and hornbeam forest edge

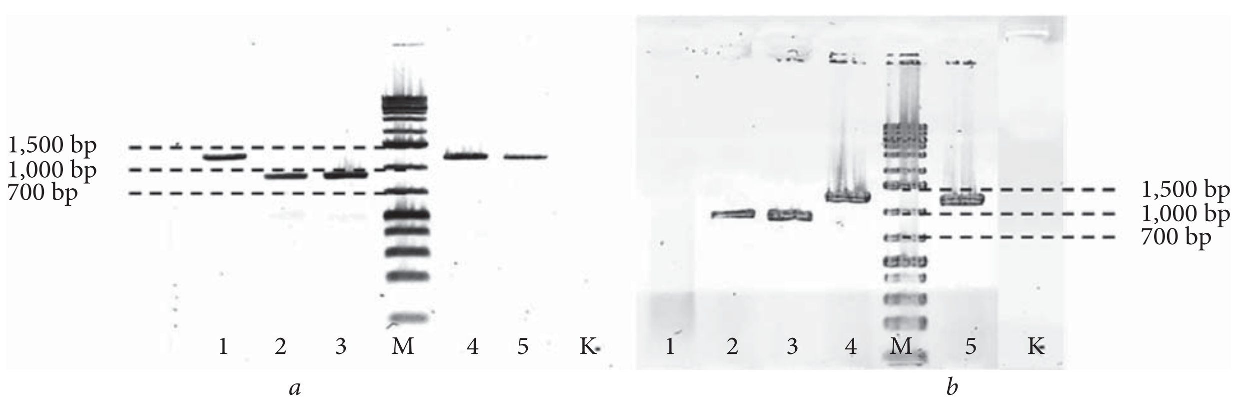

Fig. 1. Electrophoregrams of amplicons obtained by PCR: A — with DNA isolated by DNA Microprep method; B — with DNA isolated by CTAB; 1 — KY1872, 2 — KY0901, 3 — KY0881, 4 — FS0082, 5 — KY1999, M — 1 kb Plus DNA Ladder (Thermo Scientific), 75—20,000 bp; K — negative control

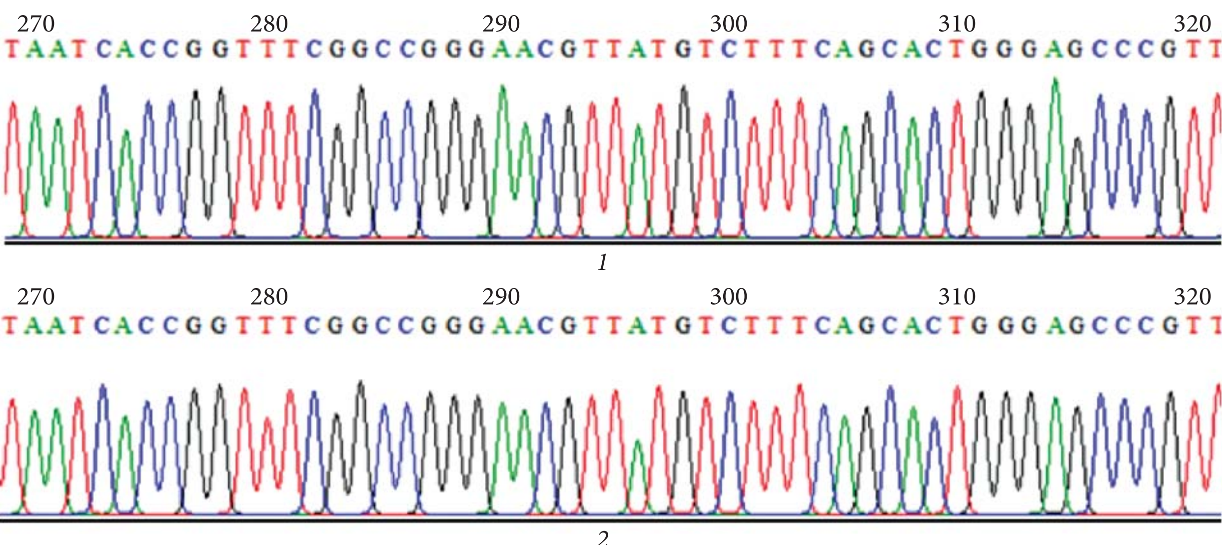

Fig. 2. Examples of successfully amplified ITS sequences chromatogram fragments from *Morchella* sp. KY1999: 1 — from DNA isolated by the DNA Microprep method; 2 — from DNA isolated by the CTAB method

Table 2. **The quantity of DNA extracted from the fruiting bodies of *Morchella* spp.**

Sample	DNA Microprep method			CTAB method		
	Concentration (ng/μL)	Purity		Concentration (ng/μL)	Purity	
		$A_{260/280}$	$A_{260/230}$		$A_{260/280}$	$A_{260/230}$
KY1872	621.382	1.95	1.92	26.412	1.82	2.02
KY0901	522.566	1.42	1.83	750.026	1.38	1.94
KY0881	199.687	2.29	2.04	221.692	1.44	1.88
FS0082	888.146	2.29	2.08	908.985	1.78	1.99
KY1999	890.448	2.06	2.02	382.800	1.53	1.88

Amplification was carried out on a Techne TC-3000X PCR thermocycler (Keison Products, UK). The initial DNA denaturation was performed at 98 °C for 3 minutes. Subsequently, 30 cycles were performed with the following parameters: denaturation at 98 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and elongation at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes. At the end of the amplification process, the temperature was lowered to 4 °C.

The amplification products were separated by electrophoresis in a 1% agarose gel.

Results. DNA-containing pellets were formed in all samples extracted by the DNA Microprep method. The DNA concentration was determined using spectrophotometry, as shown in Table 2.

As shown in Table 2, the yield of DNA extracted by the DNA Microprep method ranges from 199.687 to 890.448 ng/µL. The 260/280 ratio ranged from 1.42 to 2.29, and the 260/230 ratio — from 1.83 to 2.08. The purity of the isolated nucleic acid is acceptable, taking into account the accepted range of 260/280 (~1.8) and 260/230 (2.0—2.2) ratios (Thermo Scientific, 2011). The purity of DNA obtained by the CTAB method is slightly lower.

The amplification of the ITS marker sequence (≈ 800—1200 bp) was successful for all samples isolated by DNA Microprep method: 100% of all *Morchella* samples gave positive results in PCR (Fig. 1).

Regarding PCR products based on DNA extracted by the CTAB method, amplification was successful for 80% of samples (Fig. 1). This suggests that the DNA Microprep method is more effective for DNA extraction from morels, as it yields a sufficient amount of sufficiently pure DNA to be successfully amplified.

Since we obtained amplicons of varying lengths (≈ 800—1200 bp), potential differences in the taxonomic affiliation of the studied fruiting bodies could be inferred. To further analyze, the nucleotide sequences of the amplicons should be determined, and phylogenetic analysis should be performed. The amplicons were sent for sequencing to the Macrogen (Netherlands). The PCR products' purification was carried out on a commercial basis by Macrogen.

The resulting nucleotide sequences of ITS have been deposited in GenBank. Access codes: PP511294 (KY1872), PP582334 (KY0901), PP582336 (KY0881), PP582335 (FS0082), and PP582337 (KY1999).

Analysis of the chromatograms of the resulting nucleotide sequences showed that the quality and purity of the signal were high (Fig. 2).

Conclusions. In this study, we successfully tested the rapid and inexpensive method DNA Microprep Isolation from Plants, which could effectively reduce the time required for DNA sequencing-based molecular identification of fungi. Thus, based on the results of the study, the DNA Microprep method is generally suitable for use in DNA extraction from both fresh and herbarized morels fruit bodies. The method has a short extraction process, uses readily available reagents, is environmentally friendly, and produces fairly pure DNA. For comparison, we performed DNA extraction using a modified CTAB method. The DNA Microprep method was found to be the most successful. PCR products were obtained from the extracted DNA of the fungal samples. Subsequent sequencing of these products will enable accurate identification of species belonging to the taxonomically complex genus *Morchella*.

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Conflict of Interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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А.В. Плужник[1,2*], *В.Р. Петльована[1], В.В. Джаган[1]*

[1] Київський національний університет ім.Тараса Шевченка,
вул. Володимирська, 64/13, Київ, 01601, Україна
[2] Національний природний парк «Холодний Яр»,
вул. Холодноярська, 58, Грушківка, Черкаська обл., 20810, Україна

ШВИДКИЙ ТА ЕФЕКТИВНИЙ МЕТОД ЕКСТРАКЦІЇ ДНК
З ГРИБІВ РОДУ *MORCHELLA* DILL. EX PERS.

Таксономія грибів роду *Morchella* вже тривалий час є складною проблемою, адже ідентифікація видів такого таксономічно складного роду неможлива без використання методів молекулярної філогенетики. На сьогоднішній день існує велика кількість методів екстракції ДНК, проте пошук швидкого та ефективного методу виділення ДНК з різних організмів, зокрема з грибів, залишається актуальною проблемою сучасних біологічних досліджень. **Методи.** Матеріалом для дослідження були плодові тіла грибів роду *Morchella*. Для екстракції тотальної ДНК використовували оригінальний протокол DNA Microprep Isolation from Plants (метод DNA Microprep). Для порівняння з усіх зразків було здійснено екстракцію ДНК з використанням модифікованого CTAB-методу. Ефективність виділення встановлювали за допомогою спектрофотометрії. Ампліфікацію маркерної послідовності ITS-ділянки кластеру ядерних генів рибосомальної РНК проводили з використанням праймерів ITS1 та ITS4. Продукти ампліфікації розділяли за допомогою електрофорезу в агарозному гелі. **Результати.** ДНК-вмісна пелета утворювалась у всіх зразках, виділених за допомогою методу DNA Microprep. Успішність виділення ДНК підтверджується спектрофотометричним методом. Кількість та чистота виділеної нуклеїнової кислоти є прийнятною. Ампліфікація маркерної послідовності ITS була успішною для всіх зразків, екстрагованих DNA Microprep методом, в результаті чого отримано амплікони різної довжини, що може вказувати на потенційні відмінності в таксономічній приналежності досліджуваних плодових тіл зморшок. Щодо продуктів ПЛР на основі ДНК, екстрагованої CTAB-методом, ампліфікація була успішною для 80% зразків. **Висновки.** Результати дослідження свідчать, що метод DNA Microprep Isolation from Plants можна вважати зручним та ефективним для екстракції ДНК з плодових тіл зморшків. Перевагами методу є коротка тривалість процесу екстракції, доступність реактивів, екологічність роботи тощо. На основі виділеної ДНК було отримано ПЛР-продукти. Для точної ідентифікації видів складного в таксономічному відношенні роду *Morchella* слід встановити нуклеотидні послідовності отриманих ампліконів та здійснити філогенетичний аналіз.

Ключові слова: *екстракція ДНК, гриби, Morchella, ампліфікація ДНК, ПЛР.*

REVIEWS

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S.O. STAROVOITOVA[1,2*], Z.M. POLOVA[3], K.M. KISHKO[4], V.M. BOIKO[2]

[1] National University of Food Technologies,
68 Volodymyrska Str., Kyiv, 01601, Ukraine
[2] D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine
[3] Bogomolets National Medical University,
22 Yevhena Chykalenka Str., Kyiv, 01004, Ukraine
[4] Uzhhorod National University,
3 Narodna Square, Uzhhorod, Transcarpathian region, 88000, Ukraine
[*] Author for correspondence; e-mail: svetik_2014@ukr.net

SMALL ANIMAL MODELS OF CARDIOVASCULAR DISEASE FOR EVALUATING THE CHOLESTEROL-LOWERING ACTIVITY OF PROBIOTIC STRAINS

Cardiovascular diseases (CVDs) are major contributors to global morbidity and mortality, causing approximately 17 million deaths annually worldwide. The incidence of CVDs is rising among economically productive age groups, exacerbated by increasing rates of diabetes, obesity, and smoking-related conditions. Despite significant advances in managing low-density lipoprotein (LDL) cholesterol, the residual risk of atherosclerotic cardiovascular disease (ASCVD) persists, partly attributed to remnant cholesterol in triglyceride-rich lipoproteins. This review aims to evaluate suitable animal models of CVD for demonstrating the therapeutic efficacy of probiotic strains with cholesterol-lowering activity. Animal models that closely mimic human CVD conditions are essential for elucidating underlying disease mechanisms. Probiotics have shown promising preventive effects on CVD through the restoration of gut microbiota dysbiosis and anti-inflammatory responses. Mechanisms include reduction of oxidative stress, lowering of hypercholesterolemia, and modulation of bile acid metabolism. The advantages and limitations of animal models in CVD research are discussed, highlighting the strength of rodent models such as mice, which are cost-effective, genetically manipulable, and replicate key aspects of human CVD pathophysiology. Various contemporary mouse models are reviewed for their suitability in studying atherosclerosis, myocardial infarction, and other CVDs. Each model offers unique insights into disease mechanisms and responses to therapeutic interventions. Thus, selecting appropriate animal models is crucial for advancing our understanding of probiotic-mediated therapies in CVD. By leveraging these models, researchers can explore novel strategies to mitigate CVD risk factors and enhance therapeutic outcomes.

Keywords: *cardiovascular diseases, probiotics, animal models, atherosclerosis, cholesterol metabolism, therapeutic efficacy.*

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Cardiovascular diseases (CVDs) including heart attack, stroke, and heart failure (HF) are a leading cause of morbidity and mortality, contributing to an estimated 17 million deaths annually around the world (Wargocka-Matuszewska et al., 2023; Golforoush, et al., 2020; Savojia et al., 2019). The phenomenon is most often observed in the older age group, however, there is an increasing tendency in the economically most productive groups. Over the next few years, the incidence of CVD may multiply due to the aging of the population. The more and more common occurrence of diseases such as diabetes and obesity, as well as the conditions associated with smoking, intensifies the frequency of CVD (Aparicio et al., 2021; Amini et al., 2021; Kondo et al., 2019; Ray et al., 2022; Hansen et al., 2024). There are many underlying pathologies that lead to CVDs:

- *Atherosclerosis*. Defined by cholesterol deposition in large- and medium-sized arteries.
- *Myocardial infarction*. Unstable atherosclerotic plaque erosion leads to acute myocardial infarction, where oxygen supply to myocytes becomes restricted.
- *Arrhythmia*. Improper function of cardiac ion channels or cell junctions, or physical obstacles (e.g., infarcted tissue) disturbing electrical wave propagation.
- *Cardiomyopathy*. A general term encompassing a variety of symptoms, including heart muscle enlargement, thickening, and rigidity.
- *Cardiac fibrosis*. The scarring process characterized by cardiac fibroblast over-proliferation, myofibroblast activation, and increased deposition of fibrous extracellular matrix proteins.

For many years, low-density lipoprotein (LDL) cholesterol has been the primary lipid target for preventing cardiovascular disease, supported by extensive evidence from observational, genetic, and randomized controlled trials. Nevertheless, even after LDL cholesterol is reduced to the recommended levels, there remains a residual risk of atherosclerotic cardiovascular disease (ASCVD). This residual risk may be partly attributed to remnant cholesterol in triglyceride-rich lipoproteins. These lipoproteins are linked to an increased risk of ASCVD through both observational and genetic studies. Remnant cholesterol is calculated by subtracting LDL cholesterol and high-density lipoprotein (HDL) cholesterol from the total cholesterol. It encompasses cholesterol in very low-density lipoproteins, intermediate-density lipoproteins, and chylomicron remnants in the non-fasting state (Langsted et al., 2020; Andreadou et al., 2020).

Therefore, the **aim** of the study was to select the most correct and useable animal model of CVD to demonstrate the positive therapeutic effects of probiotic strains with cholesterol-lowering activity on these pathologies. The use of animal models recapitulating human condition is essential to understand the mechanisms underlying cardiac diseases (Savojia et al., 2019).

Current state of cardiovascular disease treatment. Disturbed cholesterol homeostasis is not only a pathological basis of cardiovascular and cerebrovascular diseases but also contributes to the progression of other conditions, including neurodegenerative diseases and cancers. Maintaining cholesterol homeostasis is crucial physiologically. Normally, cholesterol homeostasis is maintained by a dynamic balance among intake, biosynthesis, transport, cellular uptake and efflux, and esterification (Duan et al., 2022).

Currently, several treatments are recognized for their ability to lower triglyceride-rich lipoproteins:

- Statins reduce both LDL cholesterol and triglyceride-rich lipoproteins, though the percentage reduction is smaller for triglycerides compared to LDL cholesterol (Morofuji et al., 2022)
- Fibrates lower triglycerides and, in subgroup analyses of individuals with elevated triglycerides, appear to reduce the risk of atherosclerotic cardiovascular disease (ASCVD) (Duan et al., 2022)
- High doses of omega-3 fatty acids lower triglycerides (Nicholls et al., 2018)
- Inhibition of angiopoietin-related protein 3 (ANGPTL3) and apolipoprotein C-III (apoC-III) represents potential new targets. Antisense oligo-

nucleotides and antibodies have been developed to lower the plasma levels of apoC-III or ANGPTL3, thus significantly reducing plasma triglycerides (Nordestgaard et al., 2018). These drugs are expected to enter phase III trials in the near future

▪ PROMINENT trial evaluates whether reducing triglycerides and remnant cholesterol with a novel selective peroxisome proliferator-activated receptor alpha (PPARα) modulator will lower major adverse cardiovascular events (MACE) in high-risk individuals with diabetes and high triglycerides (Pradhan et al., 2018)

▪ Ezetimibe is a cholesterol absorption inhibitor used to lower total cholesterol, LDL-C, apolipoprotein B (Apo-B), and non-HDL-C in primary hyperlipidemia and familial cholesterolemia. It is a lipid-lowering compound that inhibits intestinal cholesterol and phytosterol absorption. (Khan et al., 2020)

▪ Consumption of prebiotics offers protective benefits against CVD. Among the various prebiotics, inulin and inulin-containing prebiotics have been most extensively studied for their beneficial effects on different CVDs, including coronary heart disease (CHD), diabetes associated with CHD, coronary artery disease, chronic kidney disease, atherosclerosis, and hypercholesterolemia, in both human patients and animal models (Moludi et al., 2021). Supplementation with inulin (or in combination with other components) has been shown to reduce the levels of cholesterol, including total and LDL cholesterol, C-reactive protein, and various inflammatory cytokines, while also improving antioxidant parameters and gut microbiota dysbiosis. In addition to inulin, other prebiotics and prebiotic complexes have also demonstrated beneficial roles in CVD. For instance, a prebiotic complex derived from fermented wheat bran was found to correct intestinal dysbiosis and endotoxemia in female rats with experimentally induced heart failure (Hoving et al, 2018). Soluble fiber supplementation (such as Minolest) has shown positive effects on the lipid profile of individuals with mild hypercholesterolemia and a low risk of coronary artery disease (Wu & Chiou, 2021). Moreover, larch arabinogalactan, a component of pectin, was observed to reduce myocardial injury by inhibiting apoptotic pathways in a rat model of ischemia-reperfusion. Chitosan oligosaccharides also demonstrated protective effects in CHD by enhancing antioxidant capacities and improving lipid profiles, likely by promoting the growth of beneficial probiotic species in the gut (Jiang et al, 2019). Overall, prebiotics may alleviate CVD symptoms through various mechanisms, including reducing inflammation, enhancing antioxidant capacity, and restoring balance to the dysbiotic gut microbiota.

▪ Consumption of probiotics offers protective benefits against CVD. Four mechanisms have been proposed to explain the beneficial effects of probiotics on CVD: 1) amelioration of the epithelial barrier function; 2) competition with pathogens for nutrients and adhesion sites; 3) effects on other tissues via the immune system and neurotransmitter production; and 4) immunomodulation (Sánchez et al., 2017). The preventive effects of probiotics on CVD are thought to occur through the restoration of the gut microbiota dysbiosis and anti-inflammatory responses. These mechanisms likely include reducing oxidative stress, lowering hypercholesterolemia, and decreasing high blood pressure (Oniszczuk et al., 2021). Changes in gut microbiota associated with the mediation of cholesterol metabolism, uric acid metabolism, oxidative stress, and inflammatory reactions through various metabolites could be involved in the development of atherosclerosis, a major risk factor for coronary heart disease and stroke. (Vasquez et al., 2019; Brandsma et al., 2019). In comparison with pharmaceutical agents, nutraceuticals generated from food after fermentation by probiotic bacteria have evoked greater interest in atherosclerosis prevention (O'Morain & Ramji, 2020; Jiang et al., 2020; Hassan, 2020). Probiotics play a potential role in preventing atherosclerosis through the reduction of trimethylamine N-oxide (TMAO) levels (Din, 2019; Qiu,

2018). CVDs, such as dyslipidemia and diabetes, could be caused by disorders in the metabolism of bile acids (BAs). *Lactobacilli* play an important role in BA biotransformation by promoting the activity of microbial bile salt hydrolase, regenerating primary free bile acids, and facilitating the microbial formation of secondary BAs as well as a range of intermediates (Prete et al., 2020). Obligate microflora provides hypolipidemic and hypotensive effects by deconjugating bile acids and reducing their resorption through the synthesis of specialized hydrolases; incorporating cholesterol into the lipid layer of the cell membrane; transforming cholesterol into coprostanol and removing it from the body via feces; and inhibiting cholesterol synthesis in the liver (Starovoitova et al., 2024; Hassan et al., 2019; Redinbo, 2020; Bhat et al., 2019; Daliri et al., 2022; Alaqil et al., 2020; O'Morain & Ramji, 2020; Jia et al., 2023; Asan-Ozusaglam & Gunyakti, 2019; Park et al., 2018; Bendali et al., 2017; Singhal et al., 2019; Zhang et al., 2017; Saikia et al., 2018; Bidura et al., 2019; Majeed et al., 2019; Huang et al., 2021; Haldar & Gandhi, 2019; Tom et al., 2021; Pimenta et al., 2018; Palaniyandi et al., 2020; Yusuf et al., 2020; Fernandez-Calderon et al., 2022; Dixon et al., 2020; Aswani et al., 2021; Neverovskyi et al., 2021; Pushpass et al., 2022; Frappier et al., 2022; Romero & Duarte, 2023; Taslim et al., 2023).

Thus, probiotics containing cholesterol-assimilating strains, especially in the encapsulated form (which increases the survival of probiotic microorganisms as they pass through the upper gastrointestinal tract, thereby indirectly enhancing the therapeutic effect of such treatments and products) (Starovoitova et al., 2022), can effectively complement the complex therapy of patients with cardiovascular diseases, cancer, and other conditions. These treatments are free from negative side effects associated with statins, such as hepatotoxicity; they are not addictive and do not require lifelong use. Additionally, functional foods enriched with probiotic microorganisms exhibiting hypocholesterolemic activity can be used not only in therapy but also in the prevention of diseases associated with high serum cholesterol levels (Starovoitova et al., 2024).

Pros and cons of animal models of CVD. The main advantages and limitations of animal models used in the study of cardiovascular disease are as follows:

Advantages:
- direct information;
- availability of control;
- controlled modification of variables;
- accurate techniques;
- possibility to create new models;
- availability of different models;
- greater analytical potential;
- access to structures.

Limitations:
- need to extrapolate the results;
- differences in genetic regulation;
- anatomical differences;
- various pathophysiological mechanisms;
- differences between species;
- variations induced by the techniques used;
- *in vivo* models versus *in vitro*;
- different responses to drugs.

Large animal models, such as canine and primate models, have a long history of use in CVD research. Although they effectively simulate the pathological characteristics of human patients, there are inevitable limitations, including high feeding costs and difficulties in genetic modification. Rodents, however, are the most common models for CVD research and are indispensable tools for studying the pathological features, clinical symptoms, and drug development of human diseases. Rodent models not only effectively simulate the characteristics and indicators of human CVD but also offer the advantages of strong reproductive ability and ease of detection, providing great convenience for scientific research (Table 1). Some existing rodent models for CVD are not yet fully mature or effective. More effort is needed to develop more suitable experimental rodent models for human CVDs.

Table 1. **Rodent Models and Methods for Common Cardiovascular Diseases**

Cardiovascular diseases	Modeling methods	Pros and cons
	Special diet models	
Coronary atherosclerotic heart disease	Feed apolipoprotein E deficient (Apoe−/−) mice with a high-fat diet (Gomez et al., 2018; Seijkens et al., 2018) Feed lipoprotein receptor-deficient (LDLR−/−) mice with a high-fat diet (Pan et al., 2018)	Low cost of food, simple operation Large individual differences, long experiment period
Ejection fraction-preserving heart failure	Feed Dahl salt-sensitive (DSS) rats with a high-salt diet (Cho et al., 2017)	
Coronary medial calcification	Feed dilute brown non-agouti (DBA/2) mice with a high-phosphate diet (Lau, 2013)	
Coronary intimal calcification	Feed fetuin-A/apolipoprotein E-deficient (Ahsg−/−), Apoe−/− mice with a high-phosphate diet (Jia et al., 2023)	
	Drug or immunogen-mediated models	
Low renin hypertension	Aldosterone/salt therapy (ALDOST) (Jia et al., 2023)	Significant effect, simple operation Large individual differences, risk of infecting operators
Advanced heart failure	Chronic isoproterenol stimulation (Wang, 2016)	
Rheumatic heart disease	Group A β-hemolytic streptococci (Jia et al., 2023)	
Pulmonary heart disease	Injection of monocrotaline combined with chronic hypoxic environment (Jia et al., 2023)	
Viral myocarditis	Coxsackie virus B3 (CVB3) (Remels et al., 2018; Althof et al., 2018)	
Experimental autoimmune myocarditis	α-Myosin heavy chain (α-MHC) (Miyawaki et al., 2017)	
Coronary medial calcification	Adenine (Zhou et al., 2019)	
Kawasaki disease	Water-soluble extract of *Candid albicans* (CAWS) (Miyabe et al., 2019), *Lactobacillus casei* cell wall extract (LCWE) (Jia et al., 2023)	
	Surgically induced models	
Myocardial infarction	Left anterior descending coronary artery ligation (Aghajanian et al., 2019; Park et la., 2019)	Significant effect Difficult operation, high technical requirements
Heart failure	Transverse aortic constriction (TAC) surgery (Greco et al., 2016)	
	Genetic models	
Coronary atherosclerotic heart disease	Apoe-/- mice (Jia et al., 2023)	Uniform phenotype, good controllability Long breeding time, high cost
Essential hypertension	Spontaneously hypertensive rats (SHRs) (Huc et al., 2018)	
Congenital heart disease	Nkx2-5 defect mice (mutations in the *Nkx2-5* gene are the main cause of congenital heart disease) (Furtado et al., 2017), GATA4 defect mice (the transcription factor GATA4 is a critical regulator of cardiac gene expression where it controls embryonic development, cardiomyocyte differentiation, and stress responsiveness of the adult heart. Traditional deletion of Gata4 causes embryonic lethality associated with endoderm defects and cardiac malformations, precluding an analysis of the role of GATA4 in the adult myocardium.) (Oka et al., 2006)	
Coronary artery calcification	Osteoprotegerin knockout (OPG-/-) mice (Liu et al., 2017)	
Arterial intimal calcification	Low-density lipoprotein receptor-deficient (LDLR−/−): runt-related transcription factor-2 $Runx2^{\Delta SM}$mice (Lin et al., 2016)	

Additionally, the advantages and disadvantages of small and large animal models can be discussed (Table 2) (Sosnovik & Scherrer-Crosbie, 2022; Poli et al., 2023; Liao et al., 2017; Wargocka-Matuszewska et al, 2023; von Scheidt, 2017; Golforoush, et al., 2020; Zhou et al., 2022; Hussein et al., 2023).

Since small animal models of CVD offer more advantages over large animal models, we will next examine existing small animal models for evaluating the cholesterol-lowering activity of probiotic strains and their positive effects on these diseases *in vivo*.

Contemporary small animal models of CVD. In this review, we will discuss murine models, as the mouse has become the preferred model for cardiovascular research for several reasons, including ease of handling, low procedural costs, and the ability to manipulate the mouse genome.

Mice are naturally resistant to atherosclerosis, likely because pro-atherogenic LDL-C is rapidly degraded from plasma, and the athero-protective HDL-C levels are much higher than the LDL-C levels.

Since the creation of hypercholesterolemic apolipoprotein E (apoE) gene knockout (KO) and LDL receptor (LDL-R) KO mice, which exhibit both spontaneous and diet-accelerated atherosclerosis (AS), mice have become the most widely used animal models in cardiovascular research. However, atherosclerotic plaques in mice are usually restricted to the aorta and aortic sinus, with their coronary arteries often remaining lesion-free (Sosnovik & Scherrer-Crosbie, 2022; Poli et al., 2023; Liao et al., 2017; Wargocka-Matuszewska et al., 2023; von Scheidt, 2017; Golforoush, et al., 2020; Zhou et al., 2022; Hussein et al., 2023).

LDLR−/− mice model. The LDLR−/− mouse serves as a model for familial hypercholesterolemia due to mutations affecting the LDL receptor (LDLR), with a plasma lipoprotein profile similar to that of humans. These genetically LDLR-deficient mice exhibit delayed clearance of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) from plasma. Consequently, LDLR−/− mice show a moderate increase in plasma cholesterol levels and slowly develop atherosclerosis on a normal chow diet.

Interestingly, the severity of hypercholesterolemia and atherosclerotic lesions in LDLR−/− mice can be accelerated by feeding a high-fat and

Table 2. **Advantages and disadvantages of animal models of cardiovascular diseases**

Aspects	Small animal models (rodents)	Large animal models (swine)
Advantages	Ease breading and handling	Closer to human anatomy, better tissue availability, and more accurate minimally invasive measurements
	Short reproductive cycle	Closer to human lipoprotein profile except for human HDL subclasses
	Relatively cheap	Moderately sensitive atherosclerosis on normal diet
	Well-defined genome	Vascular lesion distribution similar to that in humans
	Ease of genetic manipulation	Rare thrombosis due to plaque rupture
	Large litter number	Suitable for translational research
Disadvantages	Resistance to atherosclerosis development in Wild type (need for transgenic model)	Costly and difficult maintenance and handling
	Different gross anatomy compared to humans	No genetic modifications
	Different lipoproteins profile to humans / high level of lipid	Limited genetic models available
	Compromised lesion formation	Rare thrombosis due to plaque rupture
	Absence of plaque rupture and thrombosis	Ethical concerns

high-cholesterol diet, by mutating the apoB gene to an uneditable version, and by crossing with either leptin-deficient mice or apoB100 transgenic mice. Under these conditions, lesions in the aorta can progress beyond the foam-cell fatty streak stage to the fibroproliferative intermediate stage. The main features of the model are milder lipoprotein profile alteration compared to ApoE—/— mice and the development of atherosclerotic lesions in a time-dependent manner (Khan et al., 2018; Pan et al., 2018; Chen et al., 2024).

Apolipoprotein E knock-out mice (ApoE−/− mice) model. In 1992, two separate research groups simultaneously generated the apoE−/− mouse model by homologous recombination in embryonic stem cells. Homozygous deficiency in the apoE gene results in a marked increase in the plasma levels of LDL and VLDL due to impaired clearance through the LDLR and LDLR-related proteins. The apoE−/− mouse exhibits the entire spectrum of atherogenesis-associated lesions and is the first mouse model described to develop lesions similar to those in humans. Under normal dietary conditions, apoE−/− mice have dramatically elevated plasma cholesterol levels and develop extensive atherosclerotic lesions throughout the aorta. This process can be exacerbated by a high-fat diet, with female mice being more susceptible than males.

Chronological analysis of atherosclerotic lesions in apoE−/− mice has shown that the sequence of lesion formation is strikingly similar to that in larger animal models and humans.

Currently, apoE−/− mice are the most widely used animal model for studying atherosclerosis. Researchers have examined the effects of many genes on the development of atherosclerosis by crossing apoE−/− mice with other genetically manipulated animals. Additionally, the apoE−/− mouse serves as a valuable tool for: 1) identifying atherosclerosis susceptibility-modifying genes using a candidate-gene and gene-mapping methods; 2) deciphering molecular mechanisms and cell types involved in atherogenesis; 3) investigating the effects of drugs on atherosclerosis; 4) assessing novel therapies that prevent lesion progression. The main features of the model are spontaneously developing atherosclerosis on a normal diet; lesion progression, cell types present in the atherosclerotic plaque, and the presence of oxidized LDL, all reflecting the situation observed in humans. Notably, the apoE−/− mouse model has been used to test the additional therapeutic effects of statins beyond their cholesterol-lowering capabilities (Liao et al., 2017; Liao et al., 2021; Liu et al., 2022; Gomez et al., 2018; Seijkens et al., 2018; Chen et al., 2024).

***The LDLR and apoE double-deficient mouse* (LDLR−/−apoE−/−) model**, which develops severe hyperlipidemia and atherosclerosis even on a regular chow diet, has been proposed as a suitable model for studying the antiatherosclerotic effects of compounds without the need for an atherogenic diet. However, the response of both LDLR−/− and LDLR−/−apoE−/− mice to treatment with hypolipidemic drugs varies, ranging from lowering plasma cholesterol without reducing atherosclerosis to a weak reduction in lesions with or without decreased plasma cholesterol. In contrast, these mice respond effectively to agonists of the peroxisome proliferator-activated receptor (PPAR) or liver X receptor. This variability suggests that LDLR−/− mice may not be well-suited for analyzing the cholesterol-lowering and antiatherogenic effects of drugs (Torikai et al., 2023; Chen et al., 2024).

PCSK9 adeno-associated virus mice model. This model was developed without using germline genetic engineering. Creating this murine model required only a single injection of a recombinant adeno-associated virus (AAV) containing gain-of-function mutant forms of PCSK9, specifically human PCSK9D374Y or mouse PCSK9D377Y (AAVmPCSK9). These genes, in combination with a high-fat diet, were sufficient to reduce LDLR expression, increase plasma LDL cholesterol, and induce atherosclerosis in mice. Aortic root lesions developed in PCSK9 adeno-associated vi-

rus mice after atherosclerosis induction by a high-fat diet. An important feature of this model is its diet dependence, which also allows for the study of atherosclerosis regression. The main features of the model include the development of atherosclerosis on a fat-rich diet and the ability to study plaque calcification (Peled et al., 2017).

Mouse models of diabetes-accelerated atherosclerosis. Diabetes significantly increases the risk of CVD. Its cardiovascular complications primarily include ischemic heart disease, driven by accelerated atherosclerosis, and diabetic cardiomyopathy. To study atherosclerosis and cardiomyopathy in the context of diabetes, several mouse models are used, notably apoE−/− and LDLR−/− mice, where type 1 diabetes is induced through streptozotocin or viral injection. In these models, the induction of diabetes does not significantly alter the plasma lipid levels, effectively replicating the accelerated atherosclerosis observed in patients with type 1 diabetes (Liao et al., 2017; Liao et al., 2021; Liu et al., 2022; Gomez et al., 2018; Seijkens et al., 2018; Chen et al., 2024).

Calcium-chloride-induced abdominal aortic aneurysm* (AAA) *model. In this method, calcium chloride is applied periaortically between the renal arteries and the iliac bifurcation. Within 14 days, significant aortic dilation is observed, which becomes more severe when calcium chloride is used in conjunction with thioglycolate or the animals are fed a high-cholesterol diet. Unlike other models, this method induces AAA without requiring mechanical intervention (Krishna et al., 2020).

Elastase-induced AAA model. The elastase-induced model for AAAs in mice involves elastase perfusion in the mouse aorta, which initially causes mild-to-moderate dilation. Within 14 days, this dilation progresses to more than a 100% increase in aortic diameter. Elastase-induced injury elevates the expression of matrix metalloproteinases (MMPs), cathepsins, and other proteases, notably localizing MMP-9 to aneurysm-infiltrating macrophages. This model effectively mimics many characteristics of human AAAs, making it a valuable tool for systematically assessing the roles of individual gene products in aneurysmal degeneration. Compared to the calcium-chloride-induced AAA model, the elastase-induced method requires mechanical stress to replicate medial elastic degradation. However, it closely parallels the sequence of events seen in human AAA development (Xue et al., 2022; Wen et al., 2020).

Angiotensin II-Induced AAA model. This procedure was originally developed to determine whether elevated plasma concentrations of Angiotensin II (Ang II) directly influence the atherogenic process in aged hyperlipidemic apoE−/− mice. Ang II also induced the formation of large suprarenal AAAs in these animals. The severity of AAAs is significantly higher in hyperlipidemic apoE−/− or LDLR−/− male mice (affecting approximately 60% of mice) compared to normolipidemic mice. Here, neither hyperlipidemia nor atherosclerosis alone is considered a major determinant of aneurysm formation. This model has provided critical insights into the role of the renin-angiotensin system (RAS) in aneurysmal disease. However, two main limitations should be noted: the aneurysms in this model are located suprarenally, unlike the infrarenal location typical in humans, and the clinical relevance of RAS inhibition remains uncertain, as studies linking RAS to human AAAs have yielded controversial results (Sawada et al., 2022; Ren et al., 2022).

Spontaneous mouse mutants. The blotchy mouse strain carries a spontaneous mutation on the X chromosome, resulting in abnormal intestinal copper absorption. This defect leads to weak elastic tissue due to impaired crosslinking of elastin and collagen, causing the development of aortic aneurysms, primarily in the aortic arch and thoracic aorta, and occasionally in the abdominal aorta. However, interpreting results from this model is challenging because the mutation causes several severe additional effects beyond the aortic aneurysm formation (Liao et al., 2017; Liao et al., 2021).

Gene-targeted disruption of the muscle LIM protein (MLP) in mice serves as a model for dilated cardiomyopathy and heart failure. MLP is a regulator of myogenic differentiation. Mice homozygous for the MLP knockout develop dilated cardiomyopathy associated with myocardial hypertrophy, interstitial cell proliferation, and fibrosis. Adult mice exhibit clinical and hemodynamic signs of heart failure similar to those in humans. Due to these similarities, it has been suggested that molecular mechanisms resulting in MLP dysfunction may be involved in the development of human dilated cardiomyopathy and congestive heart failure (CHF) (Liao et al., 2017; Liao et al., 2021).

Doxorubicin cardiomyopathy. Doxorubicin exhibits both acute and chronic cardiotoxicity and has been used to induce heart failure in various animal species. Several mechanisms involved in the pathophysiology of doxorubicin-induced heart failure have been suggested, including free radical generation and lipid peroxidation, reactive sulfhydryl group interactions, binding to channel regulatory sites, and inhibition of mRNA and protein synthesis (Linders et al., 2024). It has also been shown that the suggested model (an optimal dose of doxorubicin for the simulation of congestive heart failure of 2.5 mg/animal, with a cumulative dose of 12.45 mg/kg in 4 injections every 3 days) can be used for research purposes (Spivak et al., 2013a). Moreover, gold nanoparticles of 30 nm and their AuNPs-Simdax conjugate have demonstrated positive results in biosafety and biocompatibility both *in vitro* and *in vivo*. AuNPs-Simdax and AuNPs show similar significant cardioprotective effects in rats with doxorubicin-induced heart failure, surpassing those of Simdax. Intrapleural (local) delivery is preferred over intravenous (systemic) delivery according to all tested parameters. Furthermore, sonoporation has been shown to enhance gold nanoparticle delivery to myocardial cells *in vivo* (Spivak et al., 2013b).

Murine models with Scavenger receptor class B type I (SR-BI) deficiency. SR-BI is an 85 kDa membrane glycoprotein, which contains a large extracellular domain, two transmembrane domains, a short cytoplasmic N-terminal domain, and a PDZK1-binding motif-containing C-terminal domain. Known as the primary HDL receptor with high binding affinity, SR-BI not only mediates the efflux of unesterified cholesterol (UC) from peripheral cells to circulating HDLs but also promotes the selective uptake of cholesterol esters from HDLs for biliary secretion or glucocorticoid synthesis. Modulation of its expression through the deficiency of the homologous gene or disruption of its adaptor protein PDZK1 significantly affects lipid metabolism, especially HDL metabolism. The accumulation of UC in HDLs from SR-BI-deficient mice diminishes the normal anti-atherogenic functions of HDLs. In hypercholesterolemic conditions, more UC accumulates, leading to the formation of pro-atherogenic (toxic) HDLs. Consequently, SR-BI deficiency aggravates atherosclerosis (AS). Moreover, it can even lead to occlusive coronary AS followed by spontaneous myocardial infarction in apoE KO mice on a regular chow diet (Liao et al., 2017).

SR-BI knockout and ApoE-hypomorphic mice (SR-BI KO/ApoeR61h/h mice). This model was generated by breeding two mouse strains, namely SR-BI-deficient (SR-BI KO) mice and hypomorphic apoE (ApoeR61h/h) mice, resulting in SR-BI KO/ApoeR61h/h mice. The most significant feature of the SR-BI KO/ApoeR61h/h mouse is the development of atherosclerosis and coronary heart disease in response to an atherogenic diet rich in fat, cholesterol, and cholate. This model allows investigators to control the disease onset and the severity of symptoms. It is of particular interest that due to the lack of small animal models that closely resemble the severe symptoms of atherosclerosis (formation of advanced plaques), severe coronary heart disease and even premature death are seen in humans. The main features of this model are the development of atherosclerosis and coronary heart disease on a diet rich in fat, cholesterol, and cholate,

the formation of advanced plaques, and the presence of severe coronary heart disease and premature death similar to those seen in humans. (Gonzalez et al., 2018; Burke & Huff, 2018).

Transgenic mice. Transgenic technologies have produced a range of valuable mouse models for studying hyperlipidemia and atherosclerosis. Notably, mice expressing mutant forms of apoE, such as apoE3Leiden (E3L) and apoE (Arg112→Cys142), are among the most extensively studied. These mice exhibit a lipoprotein profile similar to that of patients with dysbetalipoproteinemia, where plasma total cholesterol and triglycerides are predominantly confined to VLDL and LDL. E3L transgenic mice develop atherosclerotic lesions with characteristics akin to human vasculopathy, ranging from fatty streaks to mild, moderate, and severe plaques. Moreover, E3L transgenic mice, as well as the more recently developed E3L/Cholesteryl Ester Transfer Protein (CETP) transgenic mice, have demonstrated greater sensitivity to a variety of hypolipidemic drugs and peroxisome proliferator-activated receptor (PPAR) agonists compared to apoE−/− and LDLR−/− mice (Paalvast et al., 2017; Paalvast et al., 2022; Curry et al., 2024). For example:

- *apoE3Leiden.CETP mice*. The developed apoE3-Leiden.CETP (E3L.CETP) mouse model of atherosclerosis closely replicates the features of human disease. Among the similarities are the ability to form atherosclerotic lesions of all stages (type I to V) in a diet-induced manner and the response of diseased animals to treatments with drugs such as statins, fibrates, and ezetimibe. The model was created by combining the apoE3-Leiden transgene, which provides reduced clearance of triglyceride-rich lipoproteins, and the CETP transgene, which humanizes the cholesterol profile. The main features of the model are the formation of all stages of atherosclerotic lesions in a diet-induced manner and a human-like response to treatment with drugs such as statins, fibrates, and ezetimibe (Paalvast et al., 2017; Paalvast et al., 2022; Curry et al., 2024).
- *Apolipoprotein E-deficient fibrillin-1 mutant mice (ApoE—/—Fbn1C1039G+/— mice)*. These mice are characterized by impaired production of fibrillin-1, which leads to the fragmentation of elastic fibers observed in aortic stiffening. This condition is a potential cause of plaque rupture. The model also shares common features with atherosclerotic (apoE—/—) mice and can therefore be used to study human unstable plaques. ApoE—/—Fbn1C1039G+/— mice develop atherosclerosis in response to a high-fat diet, and this process is accelerated compared to regular apoE—/— mice. The main features of the model are the resemblance to plaque rupture and human-like complications (Curry et al., 2024).

Murine models with nitric oxide synthase (NOS) deficiency. The endothelium plays a critical role in cardiovascular health by regulating vascular tone, growth, thrombosis, and thrombolysis and inhibiting inflammation and smooth muscle cell proliferation. These functions are largely mediated by nitric oxide (NO) produced by endogenous NOS, which includes neuronal, inducible, and endothelial isoforms (nNOS, iNOS, and eNOS, respectively). eNOS is particularly important, as its dysfunction and the resulting decrease in NO production are key factors in the onset and progression of atherosclerosis.

In mice, eNOS knockout leads to elevated blood pressure variability, ejaculatory abnormalities, and impaired wound healing and angiogenesis. When eNOS knockout mice are bred with apoE knockout mice, the resulting eNOS/apoE double knockout mice develop coronary arteriosclerosis, myocardial ischemia/infarction, heart failure, and aortic aneurysms and dissections on a western diet. Despite the loss of eNOS, other NOS isoforms are upregulated, suggesting compensatory interactions within the NOS family.

To study the effects of the entire NOS system, triple knockout (tKO) mice were generated. These mice exhibited severe cardiovascular abnormalities, including hypertension, dysfunctional vascular relaxation and constriction, myo-

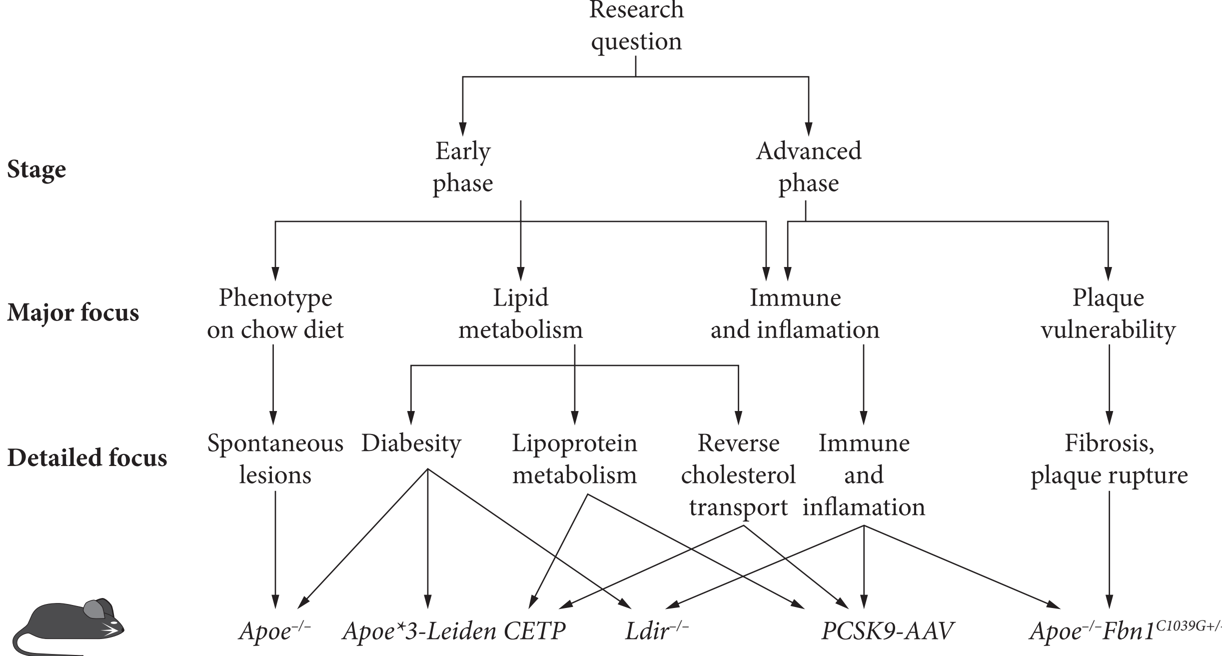

Fig. 1. Roadmap to facilitate the choice of an atherosclerotic mouse model (Oppi, 2019)

cardial infarction, left ventricular hypertrophy, and early death. While dyslipidemia is present, the coronary arteriosclerosis observed in them resembles that in eNOS/apoE double knockout mice. Additionally, significant mast cell infiltration in the coronary artery adventitia suggests that coronary spasm, potentially caused by mast cell-derived histamine release, may contribute to these conditions (Liao et al., 2021; Liao et al., 2017; Tenopoulou et al., 2018; Liu et al., 2022).

Murine models with fibrillin deficiency. Elastic fibers, composed of a cross-linked elastin core and fibrillin-rich microfibrils, are crucial for arterial elasticity and resilience. Disturbance of these fibers due to factors like aging, metabolic syndrome, and genetic defects can lead to vessel stiffness and weakness, causing conditions such as hypertension and aneurysms. Fibrillin-1, a key microfibril component, binds and sequesters growth factors and releases proteases that degrade elastin fibers. Deficiency in fibrillin-1 causes Marfan syndrome, characterized by aneurysms and skeletal defects. In apoE KO mice on a western diet, a fibrillin-1 mutation led to elastin fragmentation, accelerating atherosclerosis, intraplaque hemorrhage, and neovascularization, resulting in spontaneous plaque rupture. This caused myocardial and cerebral ischemia/infarction and death. Such mice are valuable for studying vulnerable plaque progression and therapeutic interventions (Liao et al., 2017).

Other murine models. Apart from the above strains, three other models also exhibited coronary heart disease when fed atherogenic diets. These models include apoE/LDL-R dKO mice, apoE KO mice with macrophage-targeted overexpression of urokinase, and apoE KO mice with Akt1 deficiency.

There is even a roadmap to facilitate the choice of an atherosclerotic mouse model. This scheme should help researchers choose the most appropriate atherosclerotic mouse model based on their specific research questions (Oppi, 2019) (Fig.1.).

Conclusions. This review has demonstrated that probiotics containing cholesterol-assim-

ilating strains can effectively complement the complex therapy of patients with cardiovascular diseases and other conditions associated with high serum cholesterol levels. These treatments are free from the negative side effects associated with statins, such as hepatotoxicity, and are neither addictive, nor require lifelong use.

Also discussed are the advantages and disadvantages of using small and large animals as models for CVD. Small animals, particularly mice, are commonly used in basic research on the molecular mechanisms of atherosclerosis and related cardiovascular disorders. The establishment of diet-induced CVD models has provided researchers with more options for manipulating the disease onset and progression compared to spontaneous CVD models. Despite their limitations, these animal models are invaluable tools for translational research.

Rodents are the most common models for CVD research and are indispensable for studying the pathological features, clinical symptoms, and drug development for human diseases. They effectively simulate the characteristics and indicators of human CVD and offer advantages such as strong reproductive ability and ease of detection, which provide great convenience for scientific research.

In summary, small animal models of CVD can be used to demonstrate the positive therapeutic effects of probiotic strains with cholesterol-lowering activity against pathologies *in vivo*. The use of animal models that recapitulate human conditions is essential for understanding the mechanisms underlying cardiac diseases.

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С.О. Старовойтова[1,2], Ж.М. Полова[3], *К.М. Кишко*[4], *В.М. Бойко*[2]

[1] Національний університет харчових технологій,
вул. Володимирська, 68, Київ, 01601, Україна
[2] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[3] Національний медичний університет ім. О.О. Богомольця,
вул. Євгена Чикаленка, 22, Київ, 01004, Україна
[4] «ДВНЗ» Ужгородський Національний Університет,
пл. Народна, 3, Ужгород, 88000, Закарпатська область, Україна

МОДЕЛІ СЕРЦЕВО-СУДИННИХ ЗАХВОРЮВАНЬ НА ДРІБНИХ ТВАРИНАХ ДЛЯ ОЦІНКИ ХОЛЕСТЕРИНОЗНИЖУВАЛЬНОЇ АКТИВНОСТІ ПРОБІОТИЧНИХ ШТАМІВ

Серцево-судинні захворювання є основними причинами глобальної захворюваності та смертності, спричиняючи приблизно 17 мільйонів смертей щорічно в усьому світі. Частота цих захворювань зростає серед економічно активних вікових груп, що посилюється збільшенням рівня діабету, ожиріння та захворювань, пов'язаних з курінням. Незважаючи на значний прогрес у контролі рівня холестерину ліпопротеїнів низької щільності, залишковий ризик атеросклеротичної серцево-судинної хвороби зберігається, частково через залишковий холестерин у ліпопротеїнах, багатих на тригліцериди. Цей огляд має на меті оцінити відповідні тваринні моделі серцево-судинних захворювань для демонстрації терапевтичної ефективності пробіотичних штамів, що мають здатність знижувати рівень холестерину. Тваринні моделі, які якісно імітують умови серцево-судинних захворювань у людей, є необхідними для з'ясування основних механізмів захворювання. Пробіотики показали перспективні профілактичні ефекти на серцево-судинні захворювання шляхом відновлення дисбіозу кишкової мікробіоти та протизапальних реакцій. Механізми включають зниження оксидативного стресу, зниження гіперхолестеринемії та моделювання метаболізму жовчних кислот. Обговорюються переваги та обмеження тваринних моделей у дослідженнях серцево-судинних захворювань, підкреслюючи сильні сторони гризунів, таких як миші, які є економічно вигідними, генетично маніпульованими і відтворюють ключові аспекти патофізіології серцево-судинних захворювань у людей. Розглядаються різні сучасні мишачі моделі щодо їх придатності для вивчення атеросклерозу, інфаркту міокарда та інших серцево-судинних захворювань. Кожна модель пропонує унікальне розуміння механізмів захворювання та реакцій на терапевтичні втручання. Таким чином, вибір відповідних тваринних моделей є важливим для покращення нашого розуміння терапій, опосередкованих пробіотиками, при серцево-судинних захворюваннях. Використовуючи ці моделі, дослідники можуть вивчати нові стратегії для зменшення факторів ризику серцево-судинних захворювань та підвищення терапевтичних результатів.

Ключові слова: *серцево-судинні захворювання, пробіотики, тваринні моделі, атеросклероз, метаболізм холестерину, терапевтична ефективність.*

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A.M. KYRYCHENKO[1,2*], O.H. KOVALENKO[1]

[1] Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine

[2] Institute of Biochemistry and Biophysics, Polish Academy of Sciences,
5a Pawińskiego, 02-106 Warsaw, Poland

[*] Author for correspondence: a.kyrychenko@imv.org.ua

PROSPECTS OF LIPOSOMES APPLICATION IN AGRICULTURE

Liposomes are artificially or spontaneously formed hollow structures whose contents are limited to a single, double, or multiple lipid membrane. Liposomes can be formed by amphiphilic substances encapsulating an aqueous solution of any substance under certain conditions. Liposomes have been very successfully used in the pharmaceutical, cosmetic, and food industries, but there is only limited information on the application of this technology in agriculture. Therefore, the purpose of the review is to summarize the information available since liposome discovery in the 1960s to date on the main properties of liposomes and their production technologies as well as analyze published data on the use of these supramolecular structures in agriculture, mainly as a means of storing, absorbing, and delivering pesticides or antiviral substances to plants.

Keywords: *liposomes, phospholipids, delivery systems, drug delivery, farming industries, plant resistance to viruses.*

History of liposome creation. In the mid-1960s, British hematologist Alec Douglas Bangham and his colleagues, while studying the role of phospholipids in blood clotting and the structure of dispersions formed by the swelling of phospholipids in excess water, discovered lipid vesicles, or liposomes (Bangham & Horne, 1964). Dr. Bangham realized that dried lipids spontaneously rearrange when they come into contact with a sufficient amount of water, demonstrating that this rearrangement is driven by unfavorable interactions between lipids and water, which generate repulsive effects (Trucillo et al., 2020).

In electron micrographs, Bangham observed layered particles that closely resembled cell membranes. This similarity between liposomes and cell membranes provided cell biologists with a valuable tool to study the organization, dynam-

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ics, and properties of the lipid bilayer component in biological membranes (Gregoriadis, 2016). As models of cell membranes, liposomes also allow for the study of specific membrane processes, such as fusion, membrane trafficking, cell adhesion, molecular recognition, and pore formation (Andrade et al., 2021).

Liposomes have also been used to investigate the effects of vitamins, hormones, antibiotics, and other drugs on membranes. This particular property garnered significant attention from researchers, as liposomes proved to be effective as drug carriers. Consequently, liposomes were recognized as promising systems for delivering pharmacologically active agents in the treatment of diseases (Gregoriadis et al., 1971; Gregoriadis & Ryman, 1972a, 1972b). Today, among the wide variety of drug delivery systems, the field of liposomes is one of the fastest-growing scientific topics globally. Currently, the literature on liposomes encompasses around 100,000 articles, with more than 2,000 articles published annually over the past decade. The purpose of this review was to update and summarize the key characteristics of liposomes, the methods for their creation, and the advantages of their applications across various fields.

Liposome composition. Liposomes are synthesized from a variety of components, including cholesterol, glycolipids, sphingolipids, nontoxic surfactants, long-chain fatty acids, and membranous proteins (Magar et al., 2022). The primary chemical components of liposomes are phospholipids, which may come from natural or modified natural sources, as well as semi-synthetic or fully synthetic phospholipids with modified head groups. The most commonly used phospholipids for liposome preparation are phosphatidylcholine (lecithin), phosphatidylethanolamine (cephalin), and phosphatidylserine (Trucillo et al., 2020).

When phospholipids are dispersed in water, the molecules naturally aggregate to form a bilayer, minimizing contact between the hydrophobic fatty acid chains and the surrounding hydrophilic aqueous environment. Cholesterol is a crucial lipid that significantly influences the structural properties of liposomes by reducing the permeability of the lipid bilayer. It increases the mechanical rigidity of liposomes, enhancing their stability and preventing undesirable interactions with proteins, which could otherwise impair their performance. Cholesterol also regulates phospholipid packing, membrane fluidity, and surface charge and, as a result, affects the size, morphology, and encapsulation efficiency of liposomes (Lombardo & Kiselev, 2022).

The simplest liposomes are vesicles composed of a double lipid bilayer (Fig. 1A). During the initial spontaneous reorganization of phospholipids, water-soluble drugs can be encapsulated, either within the aqueous core or within the lipid bilayer itself (Trucillo et al., 2020). More advanced liposome formulations, designed for extended circulation, incorporate polyethylene glycol (PEG) or its derivatives. These additions help reduce uptake by macrophages, thus prolonging the presence of liposomes in the bloodstream (Akbarzadeh et al., 2013).

The latest generation of liposomes is characterized by various surface modifications, such as the attachment of protective polymers, diagnostic labels, the introduction of positively charged or stimulus-sensitive lipids, as well as stimulus-sensitive polymers, cell-penetrating peptides, and viral components (Fig. 1B). These liposomes may also contain magnetic particles or gold and silver particles, which are important for magnetic targeting and imaging applications (Trucillo et al., 2020). Additionally, the surface of liposomes can be functionalized with a wide range of ligands, including monoclonal antibodies, peptides, aptamers, and growth factors. This functionalization enhances the specificity of liposome interactions, enabling targeted drug delivery and controlled drug release to specific sites, such as diseased tissues or tumors (Lombardo & Kiselev, 2022).

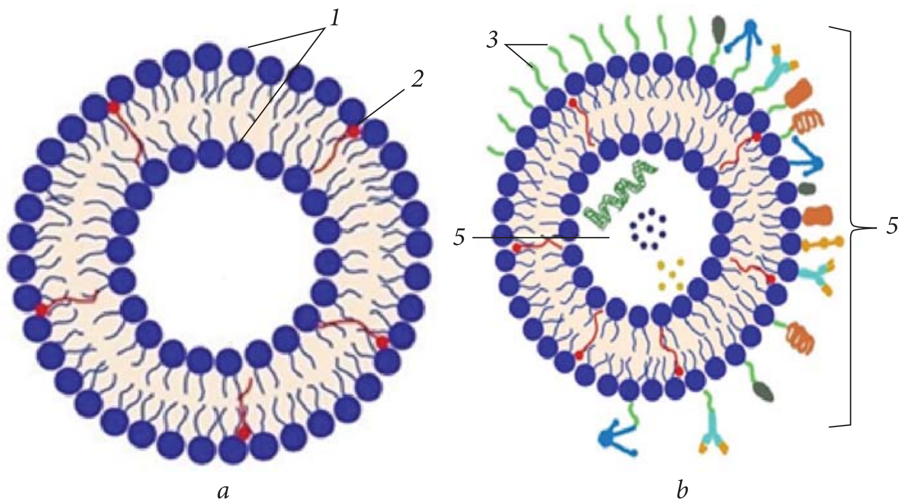

Fig. 1. Schematic representation of liposomal drug delivery systems: (*a*) Conventional liposome, (*b*) Pegylated and ligand-targeted liposome: *1* — phospholipids, *2* — cholesterol, *3* — polyethylene glycol, *4* — ligands (aptamers, charged molecules, proteins, peptides, antibodies, or other receptor-ligand bindings for site-specific targeting), *5* — hydrophilic drug (payload)

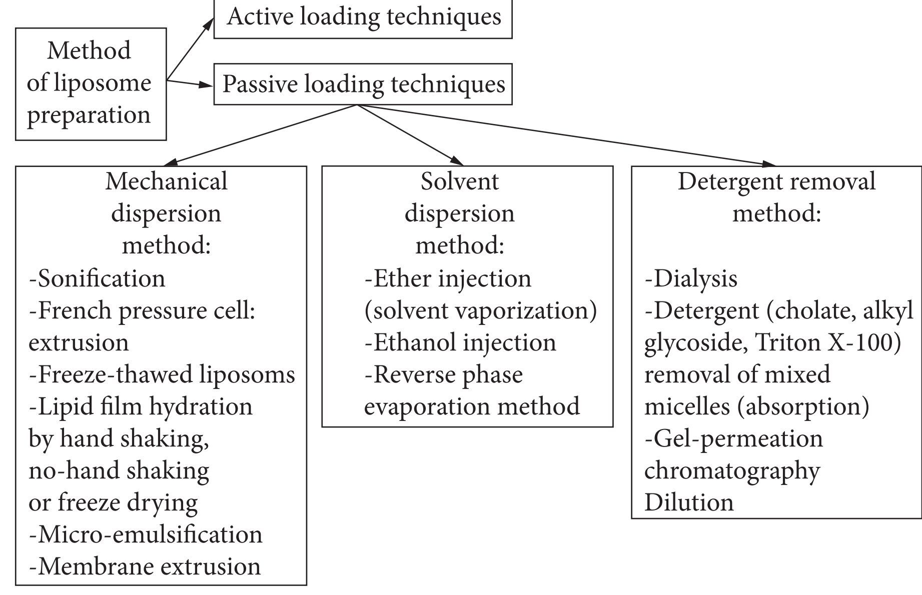

Fig. 2. Methods of liposome preparation and drug loading (according to Akbarzadeh et al., 2013)

Classification of liposomes. Liposomes are classified based on their structural parameters, preparation methods, composition, and application. Their size can range from very small (0.025 μm) to large (2.5 μm) vesicles, and they may have single or multiple bilayer membranes. Morphologically, based on vesicle size and the number of lamellae, liposomes are categorized into small (S), large (L), and giant (G) uni-, oligo-, or multilamellar vesicles (U, O, ML, respectively)

(Danilo, 2000). This classification gives rise to several widely used abbreviations:

- Small unilamellar vesicles (SUV) — 20—100 nm
- Large unilamellar vesicles (LUV) — >100 nm
- Oligolamellar vesicles (OLV) — 100—1000 nm
- Giant oligolamellar vesicles (GOV) — >1000 nm
- Multilamellar vesicles (MLV) — >500 nm.

Methods of liposome preparation. The properties of liposomes vary significantly depending on lipid composition, surface charge, size, and the method of preparation. For drug delivery, there are several established methods for preparing liposomes (Fig. 2). However, all of these methods generally follow four basic stages: (1) drying lipids from organic solvents, (2) dispersing the lipids in an aqueous medium, (3) purifying the resulting liposomes, and (4) analyzing the final product (Magar et al., 2022).

Liposome preparation typically employs either passive or active loading techniques. The passive loading technique encompasses three main approaches: solvent dispersion, mechanical dispersion, and detergent removal (for removing non-encapsulated material) (Magar et al., 2022; Akbarzadeh et al., 2013). Each method has its advantages and disadvantages. Detailed descriptions of the most commonly used liposome preparation methods have been provided in numerous reviews (New, 1990; Reineccius, 1995; Betageri & Kulkarni, 1999; Zeisig & Cämmerer, 2001; Singh et al., 2012; Akbarzadeh et al., 2013; Bozzuto & Molinфari, 2015; Lombardo & Kiselev, 2022; Magar et al., 2022; Gatto et al., 2024; Khan et al., 2024).

Liposome applications in the agricultural industry. Since their discovery in the 1960s, liposomes have been widely used as carriers for drug delivery across multiple industries, including cosmetics (Dymek et al., 2023), medicine (for diagnostics and therapy) (Gatto et al., 2024; Khan et al., 2024), and pharmacology (Akbarzadeh et al., 2013; Lian & Ho, 2001; Bulbake et al., 2017). The food industry has also explored liposomes for delivering nutrients, nutraceuticals, food additives, and antimicrobials (Singh et al., 2012; Pamunuwa & Karunaratne, 2022; Rudzińska et al., 2024). In addition, liposomes have been used successfully in such fields as oncology and gene therapy (Taylor et al., 2005; Himanshu et al., 2016; Olusanya et al., 2018; Gao et al., 2019; Gu et al., 2020; Rommasi & Esfandiari, 2021; Chavda et al., 2022).

Beyond these medical and pharmaceutical applications, liposomes have gained traction in botany and agriculture. They are used as model systems for studying cellular membranes, transmembrane metabolism in plant organelles, drug and reagent delivery systems, and for research on pollen drying tolerance and plant responses to toxins and pesticides. Liposomes have also attracted attention for their potential in encapsulating and delivering various agricultural substances.

An analysis of scientific literature over the past decade reveals a growing interest in using encapsulation technologies in agriculture, particularly for delivering pesticides (Mishra et al., 2020), fertilizers (Vejan et al., 2021), and bioactive compounds (Zabot et al., 2022). Researchers are also exploring new carriers that protect beneficial microorganisms from environmental degradation, which shows promise in advancing agricultural technologies (Jíménez-Arias et al., 2023).

Nano- and microencapsulation of active compounds offers advantages in reducing plant biotic stresses and enhancing growth by extending shelf life and enabling controlled release of biological agents. This innovative approach to managing plant pathogens and alleviating biotic stresses is thoroughly reviewed by Saberi Riseh et al. (2022).

Encapsulating beneficial microorganisms, such as mycorrhizal fungi, plant growth-promoting fungi, and plant growth-promoting rhizobacteria, as well as applying biocontrol bacteria, is considered a promising alternative to chemical fungicides. This approach helps protect

crops from phytopathogens while promoting plant growth and reducing harmful pathogens (Jamali et al., 2004; Saberi-Riseh et al., 2004). To preserve the activity of biocontrol bacteria, it is crucial to select an appropriate inoculum formulation and a carrier (Saberi-Riseh et al., 2023a).

Starch-based capsules, used to encapsulate antimicrobial agents or serve as carriers for plant-beneficial microorganisms, are effective tools against phytopathogens (Saberi-Riseh et al., 2023a). While starch has a few limitations as a primary encapsulating matrix, this can be mitigated by combining it with other biopolymers, such as lipids or polysaccharides like plant gum and carboxymethyl cellulose (CMC) (Saberi-Riseh et al., 2023b). CMC, a cellulose derivative, holds great promise for agricultural applications. Nano- and micro-CMC-based formulations for encapsulating and delivering biological control agents (BCAs) and bioactive metabolites are seen as a promising strategy for agriculture (Saberi-Riseh et al., 2022; Cokmus & Elcin, 1995; Fathi et al., 2021; Brondi et al., 2022; Saberi-Riseh et al., 2023a). The use of edible biopolymer-based delivery systems, such as those derived from natural polymers like CMC or starch, provides significant benefits in agriculture. These systems are non-toxic, biocompatible, biodegradable, and environmentally friendly, along with offering cost savings. Targeted delivery of biological and chemical pesticides in such systems improves their efficiency (Saberi-Riseh et al., 2022).

In addition to CMC and starch, research has focused on other biopolymers such as chitosan, alginate, carrageenan, xanthan, and guar gums. Despite the growing use of nanotechnology in agriculture, there has been relatively little research on using liposomes as nanocarriers (Jíménez-Arias et al., 2023). However, recent reductions in lipid costs have opened up opportunities for using liposomes in the agricultural sector. Liposomes can serve as carriers for slow-release formulations of insecticides, biostimulants, fertilizers, and nutrients, and can be used to prime plants and trigger defense responses against pathogens (Taylor et al., 2005; Shao et al., 2022).

Liposomes' ability to carry complex payloads across biological barriers and target specific tissues makes them highly effective for delivering agricultural ingredients to plants. Karny et al. (2018) demonstrated that 100-nanometer liposomes could successfully deliver active ingredients to both seedlings and fully-grown crops. Their study revealed that such liposomes penetrated the plant leaves and moved di-directionally, traveling to both roots and other leaves. Inside the plant, the liposomes released their contents using the plant's natural transport mechanisms, all without causing any toxicity. These findings underscore the potential of liposomal nanotechnology as a promising solution for enhancing plant growth and treating acute nutrient deficiencies in crops (Karny et al., 2018).

Liposomal delivery systems have shown promising results in enhancing the effectiveness of foliar-applied iron (Fe) fertilizers. In particular, liposome-encapsulated Fe (Fe-L) can efficiently deliver iron to plant tissues by overcoming cell membrane barriers. A study by Farshchi et al. (2021) demonstrated that egg-derived phosphatidylcholine (EPC)/Fe-liposomes improved plant health metrics such as fresh and dry weight, total leaf area, chlorophyll levels, ferrous content, and essential oil production compared to traditional $FeSO_4$ fertilizers. This suggests liposomal formulations could be a more efficient alternative to soil fertilization.

Despite the success of liposome technology in agriculture, its potential to boost plant resistance to viral and bacterial diseases remains largely untapped. However, promising results have emerged regarding the use of liposomes to enhance biochemical responses to biotic and abiotic stresses. Several studies have documented the priming effects of botanical extracts encapsulated in nanoscale liposomes, tested under field and lab conditions. This encapsu-

lation improves the uptake and delivery of active plant compounds, boosting plant defense mechanisms (Hegedűs et al., 2022; Kutasy et al., 2022; Decsi et al., 2023). For instance, a plant biostimulant like supercritical carbon dioxide (SC-CO_2) garlic extract encapsulated in liposomes («garlic-lipo») showed better penetration and translocation of its phytochemicals. Genome-wide data from the study indicated that liposome-delivered immunochemicals could trigger plant immune responses, resulting in healthier plants and offering a potential avenue for developing sustainable green plant protection technologies (Kutasy et al., 2022).

Nanoliposomes composed of lipid-based nanomaterials are emerging as promising tools for delivering pesticides. Wang et al. (2022) explored their use in controlling plant virus diseases by delivering antiviral agents through nanoliposome systems. Specifically, they developed a nanoliposome-based biopesticide system encapsulating quercetin, an environmentally friendly antiviral agent, creating a quercetin nano-liposome (H-TQ-NPs). Quercetin (at a concentration of 1 mol/L) effectively inhibited up to 90% of the proliferation of cucumber necrosis virus, turnip crinkle virus, and tobacco mosaic virus in *Nicotiana benthamiana* leaf cells (Wang et al., 2009).

Encapsulating quercetin in nanoliposomes enhanced its delivery into cells, improving its solubility and stability — two crucial factors for controlling phytopathogens. Quercetin's antiviral action is linked to its ability to inhibit the expression of Nbhsp70 proteins, which are upregulated during virus infections or stress and are exploited by viruses for replication. By reducing these proteins, quercetin acts as a potent antiviral agent, even under challenging field conditions (Wang et al., 2009).

The nanoliposome-encapsulated quercetin showed superior efficacy compared to free quercetin. It reduced disease symptoms, such as scorching, leaf shrinkage, and curling during viral infections. The encapsulated version provided a 33.6% and 42% increase in inhibition at the gene and protein levels, respectively, compared to free quercetin. In field experiments, the liposomal formulation demonstrated 38% higher effectiveness against the tobacco mosaic virus (TMV) than traditional methods (Wang et al., 2022). The authors assert that before their exploration of encapsulating quercetin in nanoliposomes and studying its antiviral activity, no studies had focused on using nanocarriers to deliver antiviral agents to plants. However, earlier work by Kovalenko et al. (2019) demonstrated that glycan encapsulated in liposomes formed with rhamnolipids and thiosulfonate could eliminate common bean mosaic virus from infected plant tissues, indicating early advancements in plant viral control through nanocarriers.

Nearly 20 years ago, our laboratory embarked on the development of liposomal glycans as antiviral agents. Our initial findings indicated that biopolymers demonstrated enhanced activity in the presence of surfactants (SAS) (Kovalenko et al., 2006), particularly biosurfactants such as rhamnolipids (Rh-1 and Rh-2) extracted from *Pseudomonas* sp. strain PS-17 (Kovalenko et al., 2008). These rhamnolipids served as the foundation for liposome formation, with cholesterol playing a secondary role as a donor of amino groups, facilitating the binding of carboxyl groups on the rhamnolipids. We successfully developed liposomal formulations of various glycans, including cellular glucan from *Ganoderma adspersum* (Schulzer) Donk, extracellular glucuronoxylomannan from *Tremella mesenterica* Ritz. O., and cellular mannan from *Candida maltosa* (Kovalenko et al., 2017, 2022). These resulting liposomal preparations not only exhibited prophylactic antiviral effects but also positively impacted the symbiotic properties of rhizobial microflora in legumes under field conditions (Kovalenko et al., 2022). In laboratory experiments, liposomes loaded with glucans and mannans showed antiviral activity against to-

bacco mosaic virus in virus-sensitive plants such as *Datura stramonium* L. and *Nicotiana tabacum* L. (Kovalenko et al., 2017). Additionally, our research has demonstrated for the first time that, in combination with rhamnolipids, thiosulfonates (synthetic analogs of natural biocides) can effectively serve as donors of amine groups for liposome formation. Liposomes formulated with these components and loaded with glycans exhibit highly effective antiviral activity against TMV (Kovalenko et al., 2017, 2022, 2023).

We have investigated the biological activity of these liposomal preparations on seeds of radish, tobacco, tomato, and wheat, as well as on several model plants including tobacco, wheat, and soybeans. The experimental results indicate that loading natural glycans into the liposomes significantly enhances their antiviral activity. The assessments of the phytotoxicity of these liposomal structures at concentrations effective against model viruses confirm their safety and suitability for use on plants. According to our findings, these substances facilitate the penetration of active biopolymers into plant cells with rigid cuticles and cellulose cell walls (Kovalenko et al., 2016).

These complex formulations, composed of glycans, rhamnolipids, and thiosulfonates, also functioned as effective inducers of natural plant resistance and inhibitors of TMV. The effective concentrations of these preparations as resistance inducers in *Nicotiana tabacum* var. Immune 580, which is hypersensitive to TMV, were found to be 10 and 100 μg/mL. At these concentrations, the liposomal preparations also reduce TMV infectivity in *Datura metel* L., demonstrating their role as viral infection inhibitors.

The inducing activity of these complex preparations was sensitive to actinomycin D (10 μg/mL), a known transcription inhibitor (Kovalenko et al., 2023). This observation implies that RNA synthesis plays a crucial role in activating plant virus resistance by the studied preparations. The research results highlight the potential of liposomes composed of rhamnolipids, thiosulfonates, and glycans for targeted delivery of biologically active substances, contributing to increased yields and improved crop productivity in field crops.

Conclusions. The rapidly growing global population and the consequent demand for grain and food products necessitate the search for new ways to enhance agricultural productivity. Challenges such as excessive fertilizer use, environmental pollution, plant diseases, and limited land availability require innovative approaches in crop production technology. One of the promising approaches and solutions is the use of nanotechnologies and nanomaterials in agriculture.

Utilizing biologically active substances of natural origin, which are biodegradable and environmentally friendly, can help reduce environmental pollution. Integrating biological antiviral agents, bioactive stimulants, biopesticides, and nanotechnologies offers a viable solution for combating plant diseases, supplying essential nutrients, and increasing overall crop yields.

While research on liposomal nanotechnologies in agriculture has primarily focused on controlled-release fertilizers and delivery systems for agrochemicals, their potential for crop protection against pests and pathogens remains underexplored.

Given the environmental safety and non-toxicity to humans and animals, liposomal preparations could serve as effective regulators of plant genome activity, phytohormonal status, and immune responses. These bionanomaterials hold promise not only as carriers (smart delivery systems) for agrochemicals and micronutrients but also as inducers and regulators of plant resistance. Research on the effectiveness of these preparations on plant models under field or greenhouse conditions can provide compelling evidence for the feasibility of using these nanopreparations in the agricultural sector. Future research efforts should focus on developing new

technologies for creating nanocarriers, such as liposomal preparations using components of biological origin and conducting field trials to assess their biosafety. The application of these formulations represents an environmentally friendly solution for sustainable agricultural production, preserving biodiversity in agricultural lands, and enhancing the viability and productivity of crops, all while avoiding the excessive use of traditional chemical pesticides.

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А.М. Кириченко[1,2], О.Г. Коваленко[1]

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[2] Інститут біохімії і біофізики, Польська академія наук,
5а Павінського, 02-106 Варшава, Польща

ПЕРСПЕКТИВИ ЗАСТОСУВАННЯ ЛІПОСОМАЛЬНИХ ФОРМ ЗАСОБІВ ЗАХИСТУ РОСЛИН У СІЛЬСЬКОМУ ГОСПОДАРСТВІ

Ліпосоми — це штучно або спонтанно утворені порожнисті структури, що обмежуються одно-, дво- або кількашаровою ліпідною мембраною. До утворення ліпосом здатні амфіфільні речовини, які за певних умов можуть інкапсулювати будь-які речовини з водного розчину. Ліпосоми успішно використовуються у фармацевтичній, косметичній та харчовій промисловості, однак відомості про застосування цієї технології в сільському господарстві досить обмежені. Відтак, метою цього огляду є узагальнити інформацію, доступну з часу відкриття ліпосом у 1960-х роках і дотепер, щодо основних властивостей ліпосом і технологій їх виробництва, а також аналізувати опубліковані дані з використання цих супрамолекулярних структур у сільському господарстві, в основному як засобів збереження, поглинання і доставки пестицидів та антивірусних речовин в рослинах.

Ключові слова: *ліпосоми, фосфоліпіди, системи доставки, доставка ліків, сільське господарство, вірусостійкість рослин.*