DOI: 10.14739/mmt.2024.4.311797 Оригінальні дослідження / Original research
UDC 579.864:579.83].042.2:615.33.015

# Inhibitory potential of probiotic spore formers grown as mono- and mix-cultures under conditions of different nutrient availability

**O. V. Knysh*[A-F], A. V. Martynov[A-C,E,F], T. P. Osolodchenko[B,C]**

State Institution "I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine", Kharkiv

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

**Keywords:**
A. clausii, H. coagulans, B. subtilis, S. aureus, E. coli, P. aeruginosa, inhibitory activity, dissemination ability, co-cultivation, nutrient depletion, starvation.

In many clinical cases, the use of probiotic microorganisms is a safer alternative to antibiotic therapy. Spore-forming probiotics, due to their high resistance to damaging environmental factors, stand out among other beneficial microorganisms and, therefore, deserve special attention. Determining the conditions that enhance the inhibitory potential of probiotic bacilli is an important step towards increasing their effectiveness against pathogenic bacteria and pathobionts.

**The aim** of this study was to determine whether the inhibitory potential of spore-forming probiotic species: *Alkalihalobacillus clausii* (previously known as *B. clausii*), *Heyndrickxia coagulans* (formerly *B. coagulans*) and *B. subtilis* increases under conditions of co-cultivation or nutrient depletion (starvation).

**Materials and methods.** The commercial strains of bacilli from three probiotic preparations: Enterogermina (*A. clausii*), Lactovit forte (*H. coagulans*) and Subalin (*B. subtilis*) were used in this study. The ability of mono- and mix-cultures of the studied probiotic species cultured on conventional and semi-starvation nutrient media to affect the growth of indicator bacteria-pathobionts (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*) was investigated by the agar block method (using 1.5 % nutrient agar) and the spot-on-lawn assay with wells (using 0.7 % nutrient agar).

**Results.** Inhibitory activity against indicator bacteria-pathobionts was characteristic of both mono- and mixed probiotic test cultures. Using the agar block method, probiotic monocultures grown on nutritive agar demonstrated moderate inhibitory activity against *S. aureus* and *P. aeruginosa*, but weak inhibitory activity against *E. coli.* Probiotic monocultures grown on "semi-starvation" agar and mix-cultures regardless of the culture medium showed pronounced inhibitory activity against *S. aureus* and *P. aeruginosa*, but moderate inhibitory activity against *E. coli.* Co-cultivation and cultivation on semi-starved media were accompanied by an increase in the number of isolated colonies of probiotic bacilli (disseminates) in the transparent zone of no growth and the pathobiont growth zone. Using spot-on-lawn assay revealed moderate inhibition of staphylococcus growth by both mono- and mixed probiotic cultures, regardless of their cultivation conditions. However, the inhibition indicators of probiotic mix-cultures were statistically significantly higher than those of monocultures. The inhibitory activity of probiotic monocultures against *E. coli* and *P. aeruginosa* was moderate, while probiotic mix-cultures had a pronounced inhibitory effect on these gram-negative bacteria.

**Conclusions.** The inhibitory activity and dissemination ability of spore-forming probiotic species: *A. clausii*, *H. coagulans* and *B. subtilis* increases under conditions of co-cultivation or nutrient depletion (starvation). The demonstrated effect of increasing the inhibitory potential of probiotic spore-forming bacteria by co-cultivation and application of nutrient depletion conditions requires further study and clarification of the underlying molecular mechanisms.

**Modern medical technology. 2024;16(4):310-317**

**Ключові слова:**
A. clausii, H. coagulans, B. subtilis, S. aureus, E. coli, P. aeruginosa, інгібіторна активність, здатність до дисемінації, спільне культивування, виснаження поживних речовин, голодування.

Надійшла до редакції / Received: 23.09.2024

Після доопрацювання / Revised: 11.11.2024

Схвалено до друку / Accepted: 20.11.2024

**Конфлікт інтересів:**
відсутній.

**Conflicts of interest:**
authors have no conflict of interest to declare.

***E-mail:**
knysh_oksana@ukr.net

## Інгібіторний потенціал спороутворювальних пробіотичних бактерій, вирощених як моно- та мікс-культури за умов різної доступності поживних речовин

**О. В. Книш, А. В. Мартинов, Т. П. Осолодченко**

У багатьох клінічних випадках застосування пробіотичних мікроорганізмів є безпечнішою альтернативою антибіотикотерапії. Спороутворювальні пробіотики завдяки своїй високій стійкості до шкідливих факторів довкілля вирізняються серед інших корисних мікроорганізмів, тому заслуговують на особливу увагу. Визначення умов, які посилюють інгібіторний потенціал пробіотичних бацил, є важливим кроком до підвищення їхньої ефективності проти патогенних бактерій і патобіонтів.

**Мета роботи –** визначити, чи збільшується інгібіторний потенціал спороутворювальних видів пробіотиків: *Alkalihalobacillus clausii* (раніше відомий як *B. clausii*), *Heyndrickxia coagulans* (раніше *B. coagulans*) і *B. subtilis* – за умов спільного культивування або виснаження поживних речовин (голодування).

**Матеріали і методи.** Під час дослідження використали комерційні штами бактерій із трьох пробіотичних препаратів: «Ентерожерміна» (*A. clausii*), «Лактовіт форте» (*H. coagulans*) і «Субалін» (*B. subtilis*). Досліджено

здатність моно- та мікс-культур досліджених видів пробіотиків, культивованих на звичайних і напівголодних поживних середовищах, впливати на ріст індикаторних бактерій-патобіонтів (*Staphylococcus aureus*, *Escherichia coli* і *Pseudomonas aeruginosa*) методом агарових блоків (з використанням 1,5 % поживного агару) та методом лунок на газоні (з використанням 0,7 % поживного агару).

**Результати.** Інгібіторна активність щодо індикаторних бактерій-патобіонтів властива і моно-, і змішаним пробіотичним тест-культурам. При використанні методу агарових блоків пробіотичні монокультури, вирощені на поживному агарі, характеризувалися помірною інгібіторною активністю проти *S. aureus* і *P. aeruginosa*, але слабкою щодо *E. coli*. Пробіотичні монокультури, вирощені на напівголодному агарі, та змішані культури незалежно від культурального середовища показали виражену інгібіторну активність щодо *S. aureus* і *P. aeruginosa*, але помірну щодо *E. coli*. Спільне культивування та культивування на напівголодних середовищах супроводжувалося збільшенням кількості ізольованих колоній (дисемінатів) пробіотичних бацил у прозорій зоні відсутності росту та зоні росту патобіонту. За допомогою методу лунок на газоні виявлено помірне пригнічення росту стафілококів і моно-, і змішаними пробіотичними культурами незалежно від умов культивування. Проте показники інгібування пробіотичних мікс-культур статистично достовірно вищі, ніж у монокультур. Інгібіторна активність пробіотичних монокультур проти *E. coli* та *P. aeruginosa* помірна, а пробіотичні мікс-культури мали виражену інгібіторну дію на ці грамнегативні бактерії.

**Висновки.** Інгібіторна активність і здатність до поширення спороутворювальних пробіотичних видів: *A. clausii, H. coagulans* і *B. subtilis* – зростає за умов спільного культивування або виснаження поживних речовин (голодування). Виявлений ефект збільшення інгібіторного потенціалу пробіотичних спороутворювальних бактерій шляхом спільного культивування та застосування умов виснаження поживних речовин потребує продовження вивчення та уточнення молекулярних механізмів, які лежать в його основі.

**Сучасні медичні технології. 2024. Т. 16, № 4(63). С. 310-317**

Antibiotics are undoubtedly the most effective means of fighting infectious diseases. However, given their ability to cause serious adverse effects, in some clinical cases it is advisable to use safer alternative medicines. Probiotics are one of them [1,2,3]. In addition to their antimicrobial activity, probiotics have numerous human health benefits [4]. Popular probiotic microorganisms used as natural therapeutic agents are various species of the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus, Lactococcus, Enterococcus* and *Saccharomyces* [5,6].

Spore-forming probiotic bacteria have a significant advantage over many beneficial microorganisms due to their ability to survive in such extreme environmental conditions which are lethal to vegetative cells [4,7,8,9,10]. Currently, more than 40 species of probiotic bacilli are used to treat intestinal and other diseases [11]. *Alkalihalobacillus clausii* (previously known as *Bacillus clausii*), *Heyndrickxia coagulans* (formerly *Bacillus coagulans*) and *Bacillus subtilis* are among the most comprehensively studied species and are included in most commercial spore-based probiotics [8,12]. Four strains of *B. clausii* (O/C, N/R, SIN and T), derived from a single penicillin-resistant strain *B. subtilis* ATCC 9799, are recognized as safe by the European Food Safety Authority and have been added to the Qualified Presumption of Safety (QPS) list [10,13]. The US Food and Drug Administration (FDA) has granted Generally Recognized as Safe (GRAS) status to a number of probiotic strains of *B. subtilis* and *B. coagulans*, in particular, *B. coagulans* GBI-30 6086 (GRAS Notice No. GRN 660) [14,15]. The safety of recombinant *B. subtilis* strain (UCM B-5020) has been proved *in vitro*, on animals and on healthy volunteers, and the probiotic preparation Subalin based on this strain has been approved as a medical immunobiological drug by the national authority of Ukraine [16].

These probiotic species possess immunomodulatory, anti-inflammatory, antiviral, antioxidant, anticancer, antidiabetic and hypolipidemic properties, produce a number of enzymes, vitamins, other chemicals, and exhibit direct and indirect antagonistic activity against other microorganisms [8,9,10,14,16,17,18,19]. Each probiotic microorganism has specific and sometimes unique biological properties. Preferring multi-strain / species probiotic preparations over single-strain ones is based on the idea of extending the spectrum of benefit to the host organism, obtaining additional or even synergistic effects from the combination of probiotic microorganisms [20,21]. Although some researchers argue that multi-strain mixtures are not significantly more effective than single-strain probiotics, others believe that combining probiotic microorganisms is a promising strategy to improve the efficacy and prevent side effects of probiotic therapy [12,21,22,23,24]. Experimental data have been obtained indicating a higher effectiveness of multi-strain probiotics against pathogens compared to single-strain probiotics [24,25]. However, there are few studies that shed light on the nature of the interaction of probiotic microorganisms and the mechanisms of their mutual influence, leading to an increase in their joint effect [20].

Properties of bacteria and the spectrum of substances produced by them depend both on the influence of coexisting microorganisms and on the physicochemical parameters of the environment. Determining the conditions that enhance the inhibitory potential of probiotic bacilli is an important step towards increasing their effectiveness against pathogenic bacteria and pathobionts.

## Aim

The aim of this study was to determine whether the inhibitory potential of spore-forming probiotic species: *Alkalihalobacillus clausii* (previously known as *B. clausii*), *Heyndrickxia coagulans* (formerly *B. coagulans*) and *B. subtilis* increases under conditions of co-cultivation or nutrient depletion (starvation).

## Materials and methods

The commercial strains of bacilli from three probiotic preparations were used in this study:

– four multiresistant strains of *A. clausii* ENTPro: O/C (CNCM I-276), N/R (CNCM I-274), SIN (CNCM I-275) and T (CNCM I-273) from Enterogermina (Sanofi-Aventis S. P. A., Italy, contains a mixture of spores ($2.5 \times 10^9$/5 mL);

– *H. coagulans* from Lactovit forte (Mili Healthcare, Great Britain, contains $1.2 \times 10^8$ spores/capsule);

– *B. subtilis* UCM B-5020 from Subalin (Biopharma, Ukraine, contains spores and lyophilized microbial mass of a live antagonistically active culture ($1 \times 10^9$ CFU/sachet).

Suspensions prepared from probiotic preparations were heated for 15 min at 70 °C in order to kill bacteria and activate spores. Then inoculums, containing spores of one bacillus species or mixtures of two or three bacillus species in equal concentration (~$10^9$/ml), were seeded onto the surface of nutrient agar (NA, HiMedia, India) or "semi-starvation" agar (ssNA) using the "lawn" method or inoculated into nutrient broth (NB, HiMedia, India) or "semi-starvation" broth (ssNB) in 10-ml culture tubes at a ratio of 1:10 (1 part of inoculum and 9 parts of broth) and incubated at 37 °C for 48 hours.

Such pathobionts were used as indicator bacteria: *Staphylococcus aureus* ATCC 25923 *(S. aureus)*, *Escherichia coli* ATCC 25922 *(E. coli)* and *Pseudomonas aeruginosa* ATCC 27853 *(P. aeruginosa)*. Agar precultures of indicator bacteria were obtained by cultivating overnight aerobically at 37 °C on Mueller–Hinton agar (Merck, Germany).

The inoculum from indicator bacteria was prepared by suspending a few colonies from the agar preculture in sterile saline solution (0.9 % NaCl) and adjusting the suspension to a turbidity equaling 0.5 on the McFarland scale (approximately corresponds to a cell density of ~$10^8$ cells/ml). The turbidity of the suspension was measured using the Densi-La-Meter device (Pliva-Lachema Diagnostika, Czech Republic).

The inhibitory potential of mono-, double and triple mix-cultures of probiotic *Bacillus* species against pathobionts was studied by two modified diffusion methods. The effect of probiotic bacilli cultivated on the surface of a solid nutrient medium (NA and ssNA) on the growth of indicator bacteria was studied using the agar block method [26]. The influence of probiotic bacilli grown in liquid nutrient medium (NB and ssNB) on the growth of indicator bacteria was evaluated using an optimized spot-on-lawn assay with wells [27].

**Agar block method.** The inoculum from bacilli spores of one species or double / triple mixtures was sown on the surface of the NA or ssNA by "lawn" method and cultivated at 37 °C for 48 hours. It was assumed that during cultivation, diffusion into the agar of metabolites produced by bacteria, including those with inhibitory properties, occurred. Agar blocks (cylinders with a diameter of 5 mm and a height of 3 mm) with grown test cultures were cut out in the conditions of sterility. The resulting blocks were installed on the surface of freshly seeded (by inoculum from indicator bacteria) and dried agar. Plates with agar blocks were kept at a temperature of +8 ± 2 °C for an hour for the diffusion of metabolites of the test culture from the blocks into the agar with the indicator culture and in order to avoid premature growth of the latter. Then the plates were incubated at 37 °C. The results of the experiment were taken into account after 24 hours. The inhibitory activity of the test culture against the indicator culture was assessed based on the diameter of a growth inhibition zone of the latter: weak (7–14 mm), moderate (15–24 mm); pronounced (25–35 mm) and strong (36 mm and more), as described previously [28].

**Spot-on-lawn assay.** 800 µl of inoculum from indicator preculture was mixed with 16 ml of 0.7 % soft NA and directly poured onto a plate with 1.5 % NA. The plate was dried for 50 min. Into the wells with a diameter of 10 mm, made within soft NA, were added 50 µl of mono-, double or triple mix-culture of bacilli grown in NB or ssNB. The plate was incubated at 37 °C for 24 h. The inhibitory activity of the bacilli cultures and their mixtures against the indicator cultures was evaluated based on the diameter of a growth inhibition zone as described above.

All experiments were repeated in triplicate at least three times. Data were expressed as the mean ± SD. Significant differences ($p < 0.05$) between the compared indicators were determined by performing one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons using Bonferroni adjustment.

## Results

The results of the study demonstrated the presence of inhibitory activity in both mono- and mixed probiotic test cultures against indicator cultures of pathobionts. When using the agar block method, inhibition was manifested in the expansion of probiotic cultures beyond the agar blocks *(Fig. 1, b)* and the formation of a transparent zone of no growth *(Fig. 1, c)* separating the indicator cultures of pathobionts from the probiotic ones. The appearance of isolated colonies of probiotic bacilli in the transparent zone and the pathobiont growth zone *(Fig. 1, d)* prompted the use of an additional indicator to characterize the growth of test cultures. Thus, a dissemination index was introduced, indicating the weak "+" (1–2 colonies), moderate "++" (3–5 colonies), pronounced "+++" (5–10 colonies), strong "++++" (more than 10 colonies) ability or lack "–" of ability of probiotic mono- and mix-cultures to disseminate.

The inhibitory activity and dissemination ability of probiotic cultures varied depending on their cultivation conditions. Probiotic bacteria grown as monocultures on NA were characterized by moderate inhibitory activity against *S. aureus* and weak ability to disseminate *(Table 1, Fig. 2a, b)*. There were no significant differences in inhibitory activity between the three probiotic monocultures. The indicators of antistaphylococcal activity of probiotic mix-cultures grown on NA were significantly higher than those of monocultures *(Table 1, Fig. 2c)* and corresponded to moderate (*A. clausii* + *H. coagulans* and *A. clausii* + *B. subtilis* mix- cultures) or pronounced (*H. coagulans* + *B. subtilis* and *A. clausii* + *H. coagulans* + *B. subtilis* mix-cultures) inhibition. Zones of growth inhibition of the indicator culture under the influence of mix-cultures were significantly wider due to the greater expansion of probiotic cultures beyond the agar blocks and the extension of the transparent zones of no growth. In addition, there was a significant increase in the number of disseminates, indicating a higher ability to spread of mix-cultures than monocultures.

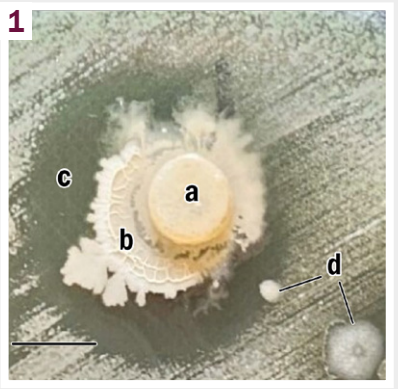


**Fig. 1.** Representative photograph of an agar block with a probiotic culture (**a**), surrounded by a zone of growth inhibition of the indicator culture (**b:** expansion of the probiotic culture beyond the agar block; **c:** transparent zone of no growth of cultures) and disseminates (**d**) – colonies of probiotic bacteria in the transparent zone / pathobiont growth zone. The scale bar is 5 mm.


**Fig. 2.** Representative photographs, demonstrating the increase in inhibitory activity and dissemination ability of probiotic bacteria under conditions of co-cultivation. Agar blocks with tested probiotic monocultures: *H. coagulans* (**2**), *B. subtilis* (**3**) and *H. coagulans* + *B. subtilis* mix-culture (**6**) on the surface of NA with *S. aureus* as indicator culture. The scale bar is 5 mm.

**Table 1.** The inhibitory activity and dissemination ability of probiotic cultures, studied using the agar block method (mean ± SD, mm)

| Test cultures | | Indicator cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | *S. aureus* | | *E. coli* | | *P. aeruginosa* | |
| | | NA | ssNA | NA | ssNA | NA | ssNA |
| Monocultures | *A. clausii* | 19.0 ± 2.4<br>+ | 25.8 ± 2.3*<br>++++ | 9.3 ± 1.8<br>+ | 15.2 ± 1.5*<br>++ | 18.0 ± 2.3<br>+ | 26.0 ± 2.3*<br>++ |
| | *H. coagulans* | 18.0 ± 3.5<br>+ | 29.3 ± 4.0*<br>++++ | 11.8 ± 1.5<br>++ | 15.7 ± 0.8*<br>+++ | 20.1 ± 2.0<br>+ | 29.8 ± 2.7*<br>++ |
| | *B. subtilis* | 17.3 ± 2.0<br>+ | 24.7 ± 3.0*<br>++ | 12.3 ± 1.4<br>+ | 15.3 ± 1.8*<br>++ | 19.0 ± 2.6<br>+ | 27.0 ± 1.8*<br>++ |
| Mix-cultures | *A. clausii* + *H. coagulans* | 24.7 ± 3.0*<br>+++ | 27.8 ± 2.3*<br>++++ | 19.2 ± 3.0*<br>++ | 17.8 ± 2.5*<br>+++ | 27.7 ± 2.2*<br>+++ | 29.0 ± 3.0*<br>++ |
| | *A. clausii* + *B. subtilis* | 24.0 ± 1.8*<br>+++ | 26.0 ± 1.4*<br>++++ | 17.8 ± 1.7*<br>++ | 17.3 ± 1.4*<br>+++ | 28.8 ± 2.9*<br>+++ | 28.1 ± 2.3*<br>++ |
| | *H. coagulans* + *B. subtilis* | 26.0 ± 3.8*<br>++++ | 26.7 ± 2.3*<br>++++ | 13.8 ± 2.6*<br>++ | 16.3 ± 0.8*<br>+++ | 28.0 ± 3.1*<br>++ | 27.2 ± 2.6*<br>++ |
| | *A. clausii* + *H. coagulans* + *B. subtilis* | 25.7 ± 3.3*<br>++++ | 27.3 ± 1.8*<br>++++ | 16.8 ± 1.7*<br>++ | 19.3 ± 1.4*<br>+++ | 29.8 ± 2.9*<br>++ | 28.9 ± 2.2*<br>++ |

***:** the differences are significant compared to the inhibitory activity of probiotic monocultures, grown on NA, $p < 0.05$.

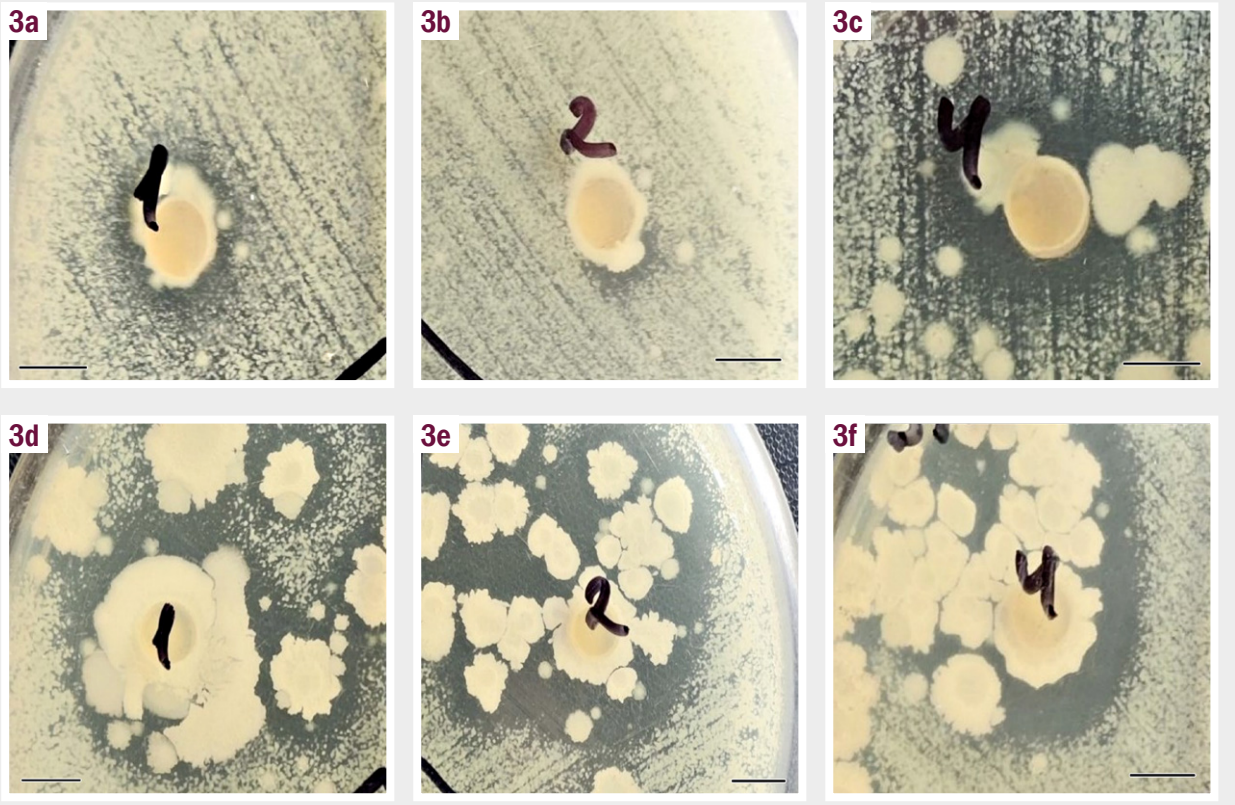


**Fig. 3.** Representative photographs demonstrating the increase in inhibitory activity and dissemination ability of probiotic bacilli under nutrient depletion (starvation) conditions. Agar blocks with tested probiotic monocultures: *A. clausii* (**1**), *H. coagulans* (**2**) and *A. clausii* + *H. coagulans* mix-culture (**4**) on the surface of NA (**a**, **b**, **c**) and ssNA (**d**, **e**, **f**) with *S. aureus* as indicator culture. The scale bar is 5 mm.

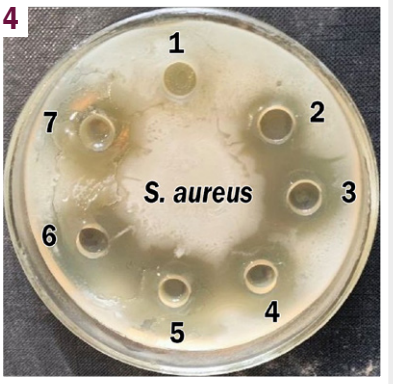


**Fig. 4.** Representative photograph, demonstrating inhibitory activity of probiotic mono- and mix-cultures: *A. clausii* (**1**), *H. coagulans* (**2**), *B. subtilis* (**3**), *A. clausii* + *H. coagulans* (**4**), *A. clausii* + *B. subtilis* (**5**), *H. coagulans* + *B. subtilis* (**6**), *A. clausii* + *H. coagulans* + *B. subtilis* (**7**) against *S. aureus*, studied by spot-on-lawn assay.

Probiotic monocultures grown on ssNA *(Table 1, Fig. 3d, e)* also showed a significantly greater inhibitory effect on the growth of the *S. aureus* indicator culture and dissemination ability than monocultures grown on NA *(Table 1, Fig. 3a, b)*. There was an increase in the expansion of probiotic monocultures beyond the agar blocks and an extension of the transparent zone of no growth to indicators corresponding to pronounced inhibition. *A. clausii* and *H. coagulans* cultures grown on ssNA had a strong, and *B. subtilis* had a moderate ability to disseminate *(Table 1)*. All mix-cultures grown on ssNA exhibited pronounced inhibitory activity and strong ability to disseminate on plates with an indicator culture of *S. aureus*. *A. clausii* + *H. coagulans* and *A. clausii* + *B. subtilis* mix-cultures grown on ssNA demonstrated higher dissemination ability than mix-cultures grown on NA *(Table 1, Fig. 3c, f)*.

Thus, the inhibitory activity against *S. aureus* and ability to disseminate of studied probiotic cultures increased when they were co-cultivated or grown in a semi-starved media.

Probiotic monocultures grown on NA had a weak inhibitory effect on the *E. coli* indicator culture *(Table 1)* mainly due to ex-

**Table 2.** The inhibitory activity of probiotic cultures, studied using spot-on-lawn assay (mean ± SD, mm)

| Test cultures | | Indicator cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | *S. aureus* | | *E. coli* | | *P. aeruginosa* | |
| | | NB | ssNB | NB | ssNB | NB | ssNB |
| Mono-cultures | *A. clausii* | 16.3 ± 2.3 | 18.3 ± 2.7 | 19.3 ± 2.9 | 19.3 ± 1.8 | 23.2 ± 3.6 | 21.9 ± 1.9 |
| | *H. coagulans* | 19.2 ± 1.9 | 19.7 ± 2.2 | 20.3 ± 1.7 | 21.2 ± 1.2 | 23.9 ± 2.7 | 24.3 ± 2.1 |
| | *B. subtilis* | 20.3 ± 2.1 | 20.7 ± 1.8 | 21.3 ± 2.2 | 21.7 ± 2.2 | 22.8 ± 2.9 | 24.0 ± 2.5 |
| Mix-cultures | *A. clausii* + *H. coagulans* | 22.7 ± 1.6* | 22.3 ± 1.6* | 24.7 ± 2.2* | 23.7 ± 1.8* | 26.4 ± 2.2* | 26.8 ± 2.5* |
| | *A. clausii* + *B. subtilis* | 24.2 ± 1.5* | 23.3 ± 2.2* | 24.3 ± 2.3* | 25.0 ± 2.0* | 28.1 ± 2.6* | 26.3 ± 2.0* |
| | *H. coagulans* + *B. subtilis* | 24.0 ± 1.8* | 23.2 ± 1.5* | 25.2 ± 2.8* | 26.0 ± 2.8* | 27.1 ± 2.5* | 27.8 ± 1.7* |
| | *A. clausii* + *H. coagulans* + *B. subtilis* | 23.3 ± 2.3* | 24.3 ± 1.6* | 26.2 ± 1.6* | 26.8 ± 2.1* | 28.0 ± 1.1* | 29.1 ± 2.6* |

***:** the differences are significant compared to the inhibitory activity of probiotic monocultures, grown in NB and ssNB, $p < 0.05$.

pansive growth beyond the agar blocks. The transparent zone of no growth of the indicator culture was barely noticeable or absent altogether. The dissemination index was low (*A. clausii* and *B. subtilis*) or moderate (*H. coagulans*). The inhibitory activity of mix-cultures grown on NA, as well as mono- and mix-cultures grown on ssNA, was significantly higher and corresponded to "moderate". However, inhibition also occurred predominantly due to expansive growth of probiotic cultures. The transparent zones of growth inhibition of the indicator culture were narrow. There were no significant differences between the inhibitory activity against *E. coli* of monocultures grown on ssNA and mix-cultures grown on both cultivation media. Co-cultivation and cultivation in a semi-starved media led to an increase in the ability of probiotic cultures to disseminate over the surface of plate with growing *E. coli* indicator culture.

Zones of growth inhibition of *P. aeruginosa* culture around agar blocks with probiotic monocultures, cultivated on NA, were consistent with "moderate inhibitory activity" in diameter *(Table 1).* However, in these zones, very weak growth of the indicator culture was observed in the form of a barely noticeable, delicate translucent coating, which indicated the bacteriostatic activity of probiotic cultures against *P. aeruginosa*. The ability of probiotic monocultures grown on NA to disseminate was weak. Cultivation of probiotic cultures on ssNA and co-cultivation on both cultivation media resulted in an increase in the diameters of growth inhibition zones of the indicator culture to sizes corresponding to "pronounced inhibitory activity", and an increase in the ability of probiotic cultures to disseminate.

The results of studying the inhibitory activity of probiotic mono- and mixed-cultures against pathobionts using the spot-on-lawn assay were in many ways similar to the results obtained using the agar block method. They confirmed that co-cultivation of probiotic bacilli leads to an increase in their inhibitory activity against pathobionts *(Table 2, Fig. 4).* However, the use of this method did not reveal significant differences between the inhibitory activity of probiotic cultures grown before sowing into wells in NB and ssNB.

## Discussion

The data obtained in this *in vitro* study clearly demonstrated the effect of enhancing the inhibitory potential of probiotic spore formers when they were co-cultivated together. The increase in inhibitory activity was manifested both in the enhanced expansion of probiotic mix-cultures beyond the agar blocks and extension of the transparent zones of no growth of the indicator culture. In addition, co-culturing of probiotic bacilli resulted in an increase in the number of their disseminates in the transparent no-growth zone and the growth zone of the indicator culture. Expansion and dissemination characterize the ability of bacteria to spread across the surface of a habitat for surface colonization. This ability is realized through various mechanisms of surface-associated motility. The use of a particular movement mechanism depends on the circumstances and involves different genes [29].

Bacilli can colonize surfaces using swimming (flagellum-mediated movement of single cells in a three-dimensional fluid space), swarming (flagellum-dependent multicellular coordinated migration in a thin liquid film on a semi-solid surface) or sliding (flagellum-independent growth-powered passive surface translocation) [29,30,31]. Under the conditions of this experiment, the spread of bacilli was possible both by swarming and sliding. The data on the increase in the ability of bacilli to spread on the surface under conditions of co-culture agree well with the results of our earlier comparative study of bacilli motility in mono- and mix-cultures and indicate the ability of bacilli to stimulate each other's motility [32]. The increased ability of probiotic cultures to spread when grown in semi-starved media is likely due to the movement of bacilli along the surface towards nutrient-rich areas with optimal conditions for growth and reproduction. The obtained data suggest that probiotic bacilli in a community, especially under starvation conditions, have a higher ability for early colonization than monocultures growing under conditions of nutrient abundance.

The direct antagonistic activity of probiotic bacilli is realized due to the antimicrobial substances, including bacteriocins (clausin, gallidermin, subtilin, etc.) and bacteriocin-like inhibitory substances (BLIS) [10,28,33,34]. Bacteriocins are a class of

allelopathic compounds produced by bacteria that can confer a competitive advantage by targeting and killing close competitors [35,36]. These antimicrobial peptides can have a bactericidal/bacteriostatic effect on both closely related and unrelated microorganisms [37,38,39]. The production of bacteriocins is metabolically demanding, so bacteria have developed mechanisms to regulate bacteriocin production depending on local conditions and especially the social environment [35,36,40].

Usually, a bacteriocinogenic strain maintains a low level of bacteriocin production until a competitor appears in its environment. Bacteriocin production increases significantly at high cell densities when competition for resources becomes high [35]. Probiotic bacteria, when co-cultured, can act toward each other as inducers of bacteriocin formation. We were prompted to this conclusion by the results of a previous study [32]. In addition, studies by several other authors have shown that co-cultivation of bacteria or bacteria with fungi can enhance the production of bacteriocins [41,42].

We also hypothesized that under conditions of nutrient deficiency, which increases competition between co-cultivated species of probiotic bacilli, their production of bacteriocins and other inhibitory substances would increase. As a result, it is possible to increase the overall inhibitory potential of mixed probiotic cultures against pathobionts and pathogenic bacteria. The results of this study confirmed our assumptions. The inhibitory activity of spore-forming probiotic species increased under conditions of co-cultivation (using both research methods) and nutrient depletion (using agar block method). The absence of the effect of increased inhibitory activity against the pathobionts of probiotic cultures grown in ssNB can be explained by the peculiarities of the research method. The spot-on-lawn assay involves the cultivation of probiotic cultures under the same conditions of nutrients availability. And presumably in 48 hours of cultivation under the same conditions, the expected difference in the inhibitory activity of probiotic cultures grown under different conditions of nutrients availability was not observed.

## Conclusions

1. The inhibitory activity and dissemination ability of spore-forming probiotic species: *A. clausii, H. coagulans* and *B. subtilis* increases under conditions of co-cultivation or nutrient depletion (starvation).

2. The demonstrated effect of increasing the inhibitory potential of probiotic spore-forming bacteria by co-cultivation and application of nutrient depletion conditions requires further study and clarification of the underlying molecular mechanisms.

**Prospects for further research**: obtained results will be applied in the further development of probiotic preparations based on consortia of spore-forming bacteria.

**Funding**

The study is a fragment of the research project of the State Institution "I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine": "Study of the stress factors influence on the antagonistic properties of spore-forming probiotic bacteria", state registration No. 0122U000422 (2022–2024).

**Information about the authors:**

Knysh O. V., MD, PhD, DSc, Senior Researcher, Leader Researcher of Laboratory and Clinical Department of Molecular Immunopharmacology, State Institution "I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine", Kharkiv.
ORCID ID: 0000-0002-4105-1299

Martynov A. V., PhD, DSc, Professor, Head of the Laboratory and Clinical Department of Molecular Immunopharmacology, State Institution "I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine", Kharkiv.
ORCID ID: 0000-0003-1428-0085

Osolodchenko T. P., PhD, Head of the Laboratory of Biochemistry and Biotechnology, State Institution "I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine", Kharkiv.
ORCID ID: 0000-0003-3589-7308

**Відомості про авторів:**

Книш О. В., д-р мед. наук, старший дослідник, провідний науковий співробітник лабораторії та клінічного відділу молекулярної імунофармакології, ДУ «Інститут мікробіології та імунології ім. І. І. Мечникова НАМН України», м. Харків.

Мартинов А. В., д-р фарм. наук, професор, зав. лабораторії та клінічного відділу молекулярної імунофармакології, ДУ «Інститут мікробіології та імунології ім. І. І. Мечникова НАМН України», м. Харків.

Осолодченко Т. П., канд. біол. наук, старший науковий співробітник, зав. лабораторії біохімії та біотехнології, ДУ «Інститут мікробіології та імунології ім. І. І. Мечникова НАМН України», м. Харків.

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