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The behavior of the *Bacillus* probiotic species under conditions of co-cultivation

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Combining probiotic bacteria is a promising strategy to increase the effectiveness and avoid side effects of probiotic therapy. Bacteria that find themselves in a common environment are able to both change their properties and show new ones under the influence of each other. The change of colonization and antagonistic properties, which provide bacteria with competitive advantages in the development of new spaces, deserves special attention.

The aim of this research was to study the features of the mutual influence of probiotic bacilli: *B. clausii, B. coagulans* and *B. subtilis* on growth, swimming and swarming motility when co-cultivated on agar media of different solidity.

Materials and methods. The study used commercial strains of bacilli from three probiotic preparations: Enterogermina, Lactovit forte and Subalin. The ability of the studied species of bacilli to influence each other's growth was investigated by the agar block method (using 1.5 % nutrient agar) and the spot-on-lawn assay (using 0.7 % nutrient agar). The study of the mutual influence on swimming and swarming activity was carried out when bacilli were cultivated on 0.25 % and 0.70 % tryptone agar, respectively. The diameters of swimming halos and migration swarms formed by mono- and mixed cultures were measured, and the areas covered by them were compared. The phenotypes of the meeting of swarms formed by cultures spotted on the swarm plates at different locations were also investigated.

Results. The studied probiotic species of the bacilli did not show a strong ability to inhibit each other's growth. The *B. clausii* culture had no inhibitory effect, and the *B. coagulans* culture demonstrated a moderate inhibitory influence on the growth of the other two species of bacilli when using both diffusion methods. The *B. subtilis* culture showed moderate or weak inhibitory activity against the *B. clausii* culture and weak or no inhibitory activity against the *B. coagulans* culture using the agar block method or spot-on-lawn assay, respectively. The *B. coagulans* + *B. subtilis, B. clausii* + *B. coagulans* and *B. clausii* + *B. subtilis* mixed cultures showed moderate, weak and no inhibitory activity against third cultures, respectively. The studied species of bacilli showed different swimming ability and swarming potential as well as the ability to influence each other's motility. Swimming halos formed by the *B. clausii* + *B. subtilis* and *B. clausii* + *B. coagulans* + *B. subtilis* mixed cultures covered significantly larger plate areas than the swimming halos formed by each culture separately during the same cultivation time. The highest swarming potential was observed in *B. coagulans* culture and *B. clausii* + *B. coagulans* + *B. subtilis* mixed culture. The studied bacilli did not show the ability to merge swarms, but, on the contrary, their swarms at the point of contact formed visible "boundary" or "intermediate" lines, demonstrating the ability to identify nonself.

Conclusions. The obtained results indicate the ability of the probiotic species of bacilli: *B. clausii, B. coagulans* and *B. subtilis* to mutually influence growth, swimming and swarm motility. The highest indicators of swimming and swarming of the triple mixed culture indicate an increase in the colonization potential of the studied bacilli when they are combined. The hypothesis of mutual induction of inhibitory compounds production by bacilli, which may increase the overall antagonistic potential of the triple mixed culture, is subject to further confirmation.

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Поведінка пробіотичних видів *Bacillus* за умов співкультивування

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Поєднання пробіотичних бактерій – перспективна стратегія підвищення ефективності й уникнення побічних ефектів пробіотичної терапії. Бактерії, що потрапили в спільне середовище, під впливом одна на одну можуть змінювати окремі властивості та виявляти нові. Особливої уваги заслуговує зміна колонізаційних та антагоністичних властивостей, що забезпечують бактеріям конкурентні переваги в освоєнні нових просторів.

Мета роботи – вивчення особливостей взаємного впливу пробіотичних бацил, а саме *B. clausii, B. coagulans* та *B. subtilis* на ріст, плавальну та ройову рухливість при спільному культивуванні на агаризованих середовищах різної густини.

Матеріали та методи. У дослідженні використовували комерційні штами бактерій трьох пробіотичних препаратів, а саме Ентерожерміна, Лактовіт форте та Субалін. Здатність видів бацил, що вивчали, взаємно впливати на ріст досліджували методом агарових блоків (з використанням 1,5 % поживного агару) та методом лунок на газоні (з використанням 0,7 % поживного агару). Взаємний вплив на плавальну та ройову активність вивчали при культивуванні бацил на 0,25 % та 0,70 % триптонному агарі відповідно. Вимірювали діаметри плавальних ореолів і міграційних зграй, утворених моно- та змішаними культурами, а також порівнювали охоплені ними площі чашок. Крім того, дослідили фенотипи зустрічі роїв, утворених культурами, що нанесені на ройових чашках у різних місцях.

Результати. Досліджені пробіотичні види бацил не виявили сильної здатності пригнічувати ріст один одного. Культура *B. clausii* не мала інгібіторного ефекту, а культура *B. coagulans* показала помірний інгібіторний вплив на ріст двох інших видів бацил; це спостерігали при використанні обох дифузійних методів. Культура *B. subtilis* характеризувалася помірною або слабкою інгібіторною активністю щодо культури *B. clausii*, не мала або виявила слабку інгібіторну активність щодо культури *B. coagulans* при застосуванні методу агарових блоків або лунок на газоні відповідно. Змішані культури *B. coagulans* + *B. subtilis, B. clausii* + *B. coagulans* і *B. clausii* + *B. subtilis* характеризувалися помірною та слабкою інгібіторною активністю відповідно, щодо третіх культур її не виявили. Досліджені види бацил характеризувалися різною плавальною активністю та потенціалом роїння, а також мали здатність впливати на рухливість один одного. Плавальні ореоли, утворені змішаними культурами *B. clausii* + *B. subtilis* та *B. clausii* + *B. coagulans* + *B. subtilis*, охоплювали значно більші площі чашок, ніж плавальні ореоли, що утворені кожною культурою окремо протягом однакового часу культивування. Найвищий потенціал до роїння визначили в *B. coagulans* та змішаної культури *B. clausii* + *B. coagulans* + *B. subtilis.* Бацили, що вивчали, не виявляли здатності до злиття роїв – їхні рої в місці контакту утворювали видимі «межові» або «проміжні» лінії, а отже мали здатність ідентифікувати «не себе».

Висновки. Результати свідчать про здатність пробіотичних видів бацил, зокрема *B. clausii, B. coagulans* і *B. subtilis*, взаємно впливати на ріст, плавання та ройову рухливість. Найвищі показники плавання та роїння потрійної мікс-культури свідчать про підвищення колонізаційного потенціалу досліджених бацил при їх поєднанні. Гіпотеза про взаємну індукцію бацилами продукції інгібіторних сполук, що може збільшити загальний антагоністичний потенціал потрійної змішаної культури, потребує підтвердження.

Сучасні медичні технології. 2023. № 4(59). С. 59-69

Probiotic bacteria of the genus *Bacillus* are of great interest to researchers. Beneficial bacilli play a key role in intestinal homeostasis, promoting the growth of other beneficial bacteria and inhibiting the growth of pathogens and pathobionts, contribute to the proper development, maturation and functioning of the immune system, strengthening the epithelial barrier, normalizing digestion and metabolism, etc. [1,2,3,4]. The former notion of *Bacillus* as "transient" members of the gut microbiome is incorrect as they are most likely intestinal colonizers [3]. Due to the high resistance of spores to adverse environmental factors, bacilli survive in stressful conditions of the gastrointestinal tract and reach the intestine without significant quantitative and qualitative losses [5,6]. This allows bacilli to realize their probiotic potential more efficiently than non-spore-forming bacteria [7,8,9,10]. Unlike vegetative probiotic bacteria of the genera *Lactobacillus* and *Bifidobacteria*, representatives of the genus *Bacillus* do not require lyophilization, as they can be stored in spore form for a long time without loss of germination [8,11].

B. clausii, B. coagulans and *B. subtilis* are some of the most commonly used commercial *Bacillus* probiotic species [10]. In addition to common probiotic activity, each species / strain has inherent only to its properties and the mechanisms of action due to the uniqueness of the structure and spectrum of produced biologically active metabolites [6,12,13,14,15]. For example, recombinant probiotic strain *B. subtilis* UCM B-5020 is capable of synthesizing human leukocyte α2-interferon due to the artificial introduction of plasmid DNA containing the interferon gene into its genome [14].

Combining probiotic species, strains, and postbiotic products is a promising strategy to improve efficacy and avoid side effects of probiotic therapy [16,17,18,19]. Multi-strain probiotics have demonstrated higher antagonistic activity against pathogens than single-strain probiotics [19,20]. Bacteria that find themselves in a common environment establish certain relationships with each other. A number of studies have established that intermicrobial interactions in a coexistence environment can lead not only to a change in the properties inherent in bacteria, but also to the emergence of new properties that are absent in a monoculture [21,22]. The study of the mutual influence of probiotic bacteria on colonization and antagonistic properties, which provide them with competitive advantages in the development of new spaces and the improvement of the microecological environment, deserves special attention.

Aim

The aim of this research was to study the features of the mutual influence of probiotic bacilli: *B. clausii*, *B. coagulans* and *B. subtilis* on growth, swimming and swarming motility when co-cultivated on agar media of different solidity.

Materials and methods

This research was carried out in the Laboratory and Clinical Department of Molecular Immunopharmacology of the State Institution "I. Mechnikov Institute of Microbiology and Immunology of the National Academy of Medical Sciences of Ukraine".

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

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

2) Lactovit forte (Mili Healthcare, Great Britain), contains 1,2 × 10^8 spores/capsule of *B. coagulans*;

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

Before inoculation, aqueous suspensions of spores (~10^9/ml) were heated for 15 min at 70 °C in order to activate spores and kill bacteria. Vegetative cells were obtained by germinating spores on the surface of nutrient agar (NA, HiMedia, India) at 37 °C for 24 hours. The inoculum 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 of 4.0 McFarland (approximately corresponds to a cell density of 1.6 ± 0.5 × 10^8 cells/ml). The turbidity of the suspension was measured using the Densi-La-Meter device (Pliva-Lachema Diagnostika, Czech Republic).

The influence of probiotic species of bacilli on the growth of each other under the conditions of co-cultivation was studied by two modified diffusion methods: agar block method and spot-on-lawn assay [23,24].

Agar block method. The inoculum from test preculture was sown on the surface of the NA by the "lawn" method and cultivated at 37 °C for 24 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 containing vegetative cells of the indicator culture) 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 by the size of the growth inhibition zone of the latter: + – weak, 1–2 mm; ++ – moderate, 2–4 mm; +++ – strong, more than 4 mm, as described previously by I. Khatri et al. [8].

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. In the center of the plate, three wells with a diameter of 10 mm were made at a distance of at least 2.5 mm from each other. 50 µl of inoculums from two test precultures and their mixture (1:1) were added to the wells. The plate was incubated at 37 °C for 24 h. The inhibitory activity of the test cultures and their mixture against the indicator culture was evaluated based on the inhibition zone as described above.

Motility assays. Swimming and swarming motility were studied in accordance with the previously described methods [25,26]. Plates with a diameter of 90 mm were used. For each experiment swim plates (TrM: 1.00 % tryptone, 0.50 % NaCl, 0.25 % agar) and swarm plates (TrA: 1.0 % tryptone, 0.5 % NaCl, 0.7 % agar) were prepared fresh daily and left overnight at room temperature before use. 5 µl of inoculum from preculture (or mixture of two / three precultures) was spotted onto the center of TrM plate. The growth halo diameter was measured every 2 hours for 10 hours and after 15 hours incubation of plates at 37 °C in a humidified chamber.

Swarming motility was initiated by spotting 50 µl of inoculum from preculture (or mixture of two/three precultures) onto the center of TrA plate. Swarming migration was evaluated by measuring swarm diameter every 2 hours for 16 hours and after 24 hours incubation of plates at 37 °C in a humidified chamber. Swarming differentiation was confirmed by visualizing the presence of elongated swarmer cells at the colony rim (*Fig. 4D*).

A swarm boundary assay was performed as described by P. Stefanic et al. [27]. To investigate difference between approaching swarms, cell suspensions of monocultures were spotted on the TrA plate at different locations. After drying for 5 minutes plates were incubated at 37 °C in a humidified chamber and photographed. The phenotypes of the meeting of swarms were determined from the photos.

All experiments were performed in triplicate at least three times. Data were statistically processed using LibreOffice Calc 7.5 OpenLicense MPL 2.0 and expressed as the mean ± standard deviation (SD). Significant differences ($p < 0.05$) between the compared indicators were determined by performing a one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons using Bonferroni adjustment.

Results

The study of relationships between bacilli on a solid nutrient medium using the agar block method showed their complex nature. The *B. clausii* culture did not show an inhibitory effect on the growth of the *B. coagulans* culture (*Fig. 1a*). Visually, the growth zone of one culture smoothly passed into the growth zone of another. Between the agar blocks with the *B. clausii* culture and the growth zones of *B. subtilis*, very thin (up to 0.5 mm) stripes of the absence of culture growth were observed, which indicated an insignificant effect of the test culture on the growth of the indicator culture (*Fig. 1b*).

On plates with the *B. clausii* indicator culture, a noticeable, up to 1.8 ± 0.8 mm, expansion of the *B. coagulans* culture beyond the agar blocks was observed (*Fig. 1c*). These two cultures were separated by 0.3 ± 0.2 mm wide streaks of no growth. Therefore, the *B. coagulans* culture has a moderate (++) inhibitory effect on the growth of the *B. clausii* culture. On plates with the *B. subtilis* indicator culture, in addition to the expansion of the *B. coagulans* culture beyond the agar blocks for a distance of up to 0.9 ± 0.4 mm, a significant growth inhibition of the indicator culture was observed (*Fig. 1d*). The width of the growth inhibition zone of *B. subtilis* culture was 2.9 ± 0.4 mm (++, moderate inhibitory activity).

On plates with the *B. clausii* indicator culture, the expansion of the *B. subtilis* culture beyond the agar block to a distance of up to 1.2 ± 0.4 mm was observed (*Fig. 1e*). The width of the

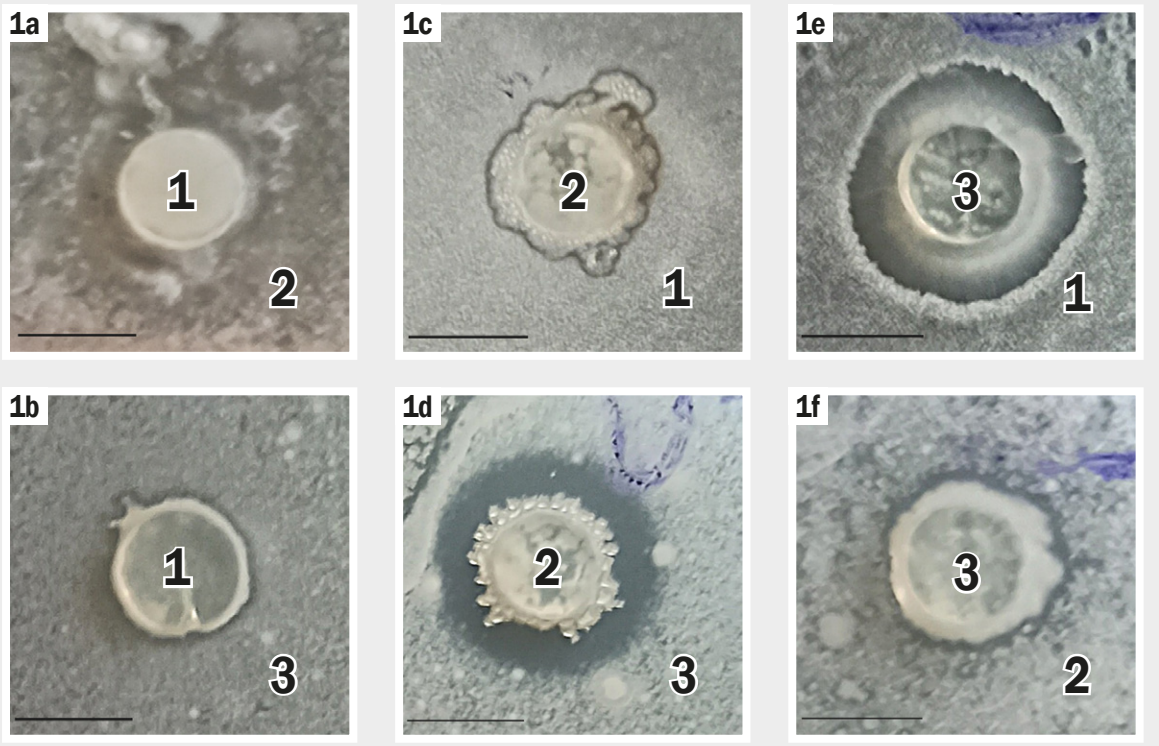


Fig. 1. Representative photographs of agar blocks with studied cultures surrounded by growth zones / growth inhibition zones of indicator cultures. **1:** *B. clausii*; **2:** *B. coagulans*; **3:** *B. subtilis*. The scale bar is 5 mm.

Table 1. The ability of probiotic *Bacillus* species to inhibit each other's growth (spot-on-lawn assay)

Test cultures	The growth inhibition zones of the indicator cultures (mean ± SD, mm)		
	B. clausii	***B. coagulans***	***B. subtilis***
B. clausii	–	<1.0	<1.0
B. coagulans	2.5 ± 0.5	–	2.1 ± 0.7
B. subtilis	1.2 ± 0.4	<1.0	–
B. coagulans + *B. subtilis*	2.9 ± 0.7	–	–
B. clausii + *B. subtilis*	–	<1.0	–
B. clausii + *B. coagulans*	–	–	1.1 ± 0.7

growth inhibition zone of the indicator culture was 1.43 ± 0.40 mm. Thus, the *B. subtilis* culture revealed a moderate (++) inhibitory effect on the growth of the *B. clausii* culture. On plates with the *B. coagulans* indicator culture, the expansion of *B. subtilis* beyond the boundaries of the agar blocks up to 1.0 ± 0.5 mm was observed (*Fig. 1f*). The *B. subtilis* and *B. coagulans* cultures were separated by a very narrow, up to 0.40 ± 0.15 mm, stripe of no growth. So, *B. subtilis* showed weak (+) inhibitory activity against *B. coagulans* culture.

The results of the spot-on-lawn assay in many ways resembled the results of the agar block method. The *B. clausii* test culture had no inhibitory effect on the growth of *B. coagulans* and *B. subtilis* indicator cultures (*Table 1*).

The *B. coagulans* test culture showed a moderate inhibitory effect on the growth of *B. clausii* and *B. subtilis* indicator cultures. A distinctive feature was that the *B. subtilis* test culture had a weak inhibitory effect on the growth of the *B. clausii* indicator culture and had no significant effect on the growth of the *B. coagulans* indicator culture. The *B. coagulans* + *B. subtilis* mixed culture showed a moderate inhibitory effect on the *B. clausii* indicator culture. The *B. clausii* + *B. coagulans* mixed culture had a weak inhibitory effect on the *B. subtilis* indicator culture. The *B. clausii* + *B. subtilis* mixed culture did not significantly affect the growth of *B. coagulans* indicator culture.

It should be noted that the studied probiotic species of bacilli did not show a strong ability to inhibit each other's growth. Schematically, the nature of intermicrobial relationships between the studied bacilli is shown in *Fig. 2*.

The aim of the next stage of the study was to find out whether the motile activity of bacilli changes during co-cultivation. The

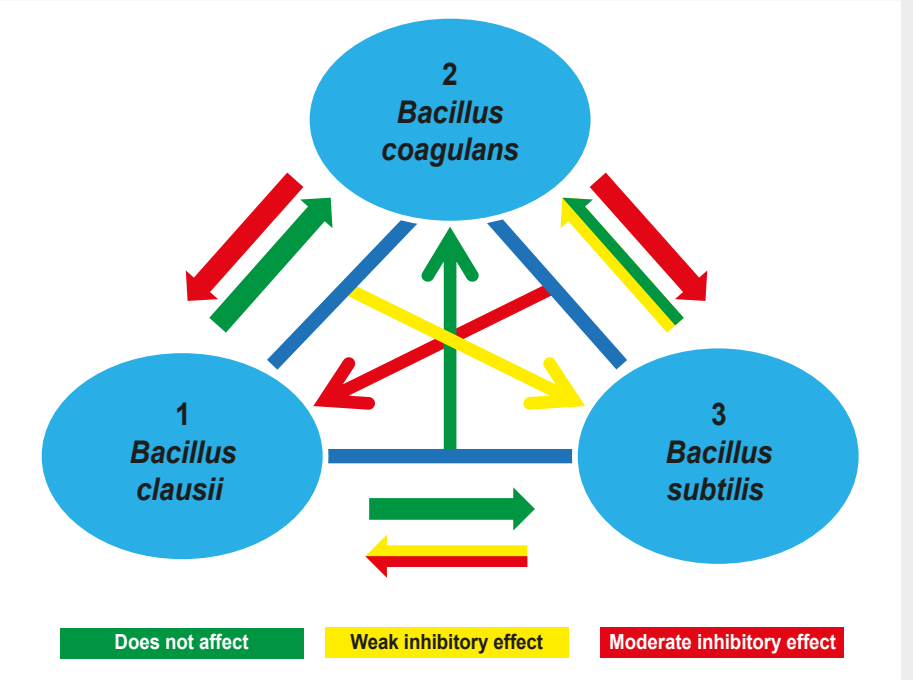


Fig. 2. Effect of probiotic bacilli on each other's growth.

studied species of bacilli showed different swimming motility (*Fig. 3A, B*). First, the bacilli had different durations of the initial lag phase. Second, the growth rate of swimming halos has been uneven over time and varied across cultures. As a result of the first and second, it took different times to completely cover the surface of the plate by swimming halos of different cultures and mixed cultures.

A short initial lag phase lasting 2.5 ± 0.5 hours was typical for the *B. coagulans* culture. After that, the diameter of the swimming halo increased at an average rate of ~10.8 mm/h. After 10 hours of cultivation, the swimming halo occupied 80.9 ± 8.0 % of the plate surface (*Fig. 3A*). The initial lag phases of the *B. clausii* and *B. subtilis* cultures lasted 5.00 ± 0.50 and 4.50 ± 0.25 hours, respectively. Then the swimming halo of the *B. clausii* culture expanded over the surface of the plate at an average rate of ~9.0 mm/h, occupying 24.9 ± 7.8 % of its area after 10 hours of cultivation. The swimming halo of the *B. subtilis* culture spread over the surface of the plate at an average rate of ~9.6 mm/h, occupying 34.7 ± 3.9 % of its area after 10 hours of cultivation.

The initial lag phase of the *B. clausii* + *B. coagulans* mixed culture lasted up to 2.50 ± 0.25 hours. The average rate of their swimming halo spreading over the plate surface was ~11.3 mm/h. After 10 hours of cultivation its area reached 89.2 ± 8.4 %, significantly exceeding the area of the swimming halo formed by *B. clausii culture* and not significantly different from the swimming halo formed by *B. coagulans* for the same cultivation time (*Fig. 3A*). The swimming halo of the *B. clausii* + *B. subtilis* mixed culture began to grow after a 4.5-hour initial lag phase at an average rate of ~11.2 mm/h. This mixed culture covered 47.5 ± 6.1 % of the plate surface after 10 hours of cultivation, which was significantly more compared to the areas occupied by the swimming halos of each culture separately. Covering the surface of the plate by the swimming halo formed by *B. coagulans* + *B. subtilis* mixed culture occurred at an average rate of ~9.3 mm/h, except for a 3-hour initial lag phase. After 10 hours of cultivation it covered the plate surface by 52.2 ± 9.6 %, which was significantly larger than the area of the *B. subtilis* swimming halo and significantly less than the area of the *B. coagulans* swimming halo. The initial lag phase of 3.0 ± 0.2 hours and the highest rate (~12.6 mm/h) of plate surface coating by a swimming halo were observed in the triple mixed culture of bacilli (*B. clausii* + *B. coagulans* + *B. subtilis*). After 10 hours of cultivation 95.6 ± 5.4 % of the plate surface was covered by this swimming halo.

The bacilli of the studied species showed different swarming abilities. The most powerful swarm potential among them was demonstrated by the *B. coagulans* culture (*Fig. 4A*). The weakest swarming potential was possessed by the *B. clausii* culture. The shortest initial lag phase of 2.00 ± 0.25 hours was observed in the *B. coagulans* culture. The initial lag phases of *B. clausii* and *B. subtilis* cultures lasted 4.50 ± 0.50 and 4.25 ± 0.25 hours, respectively. The lag phase was followed by a phase of swarm expansion. The swarm expansion rate was uneven and gradually decreased over time. The average swarm expansion rates calculated for *B. clausii, B. coagulans* and *B. subtilis* cultures were ~3.8 mm/h, ~6.2 mm/h and ~6.4 mm/h, respectively. At 16 hours after inoculation, the swarm formed by the *B. coagulans* culture covered the plate surface by 93.4 ± 6.4 %, and the swarm formed by the *B. subtilis* culture covered it by 69.4 ± 9.2 %. The swarm formed by the *B. clausii* culture during this time occupied a limited area around the inoculation point, which accounted for 23.9 ± 6.5 % of the plate surface.

When *B. clausii* or *B. subtilis* were co-cultivated with *B. coagulans* on swarm plates, the initial lag phases were reduced

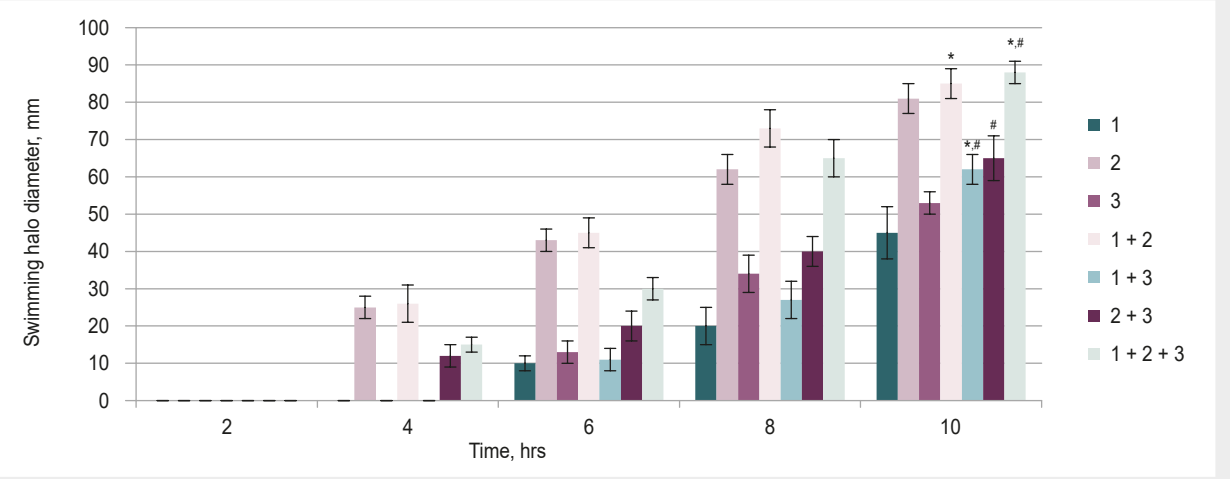


Fig. 3A. Swimming ability of *Bacillus* probiotic species under conditions of mono- and co-cultivation.
***:** the differences are significant compared to sample 1; **#:** the differences are significant compared to sample 3, $p < 0.05$

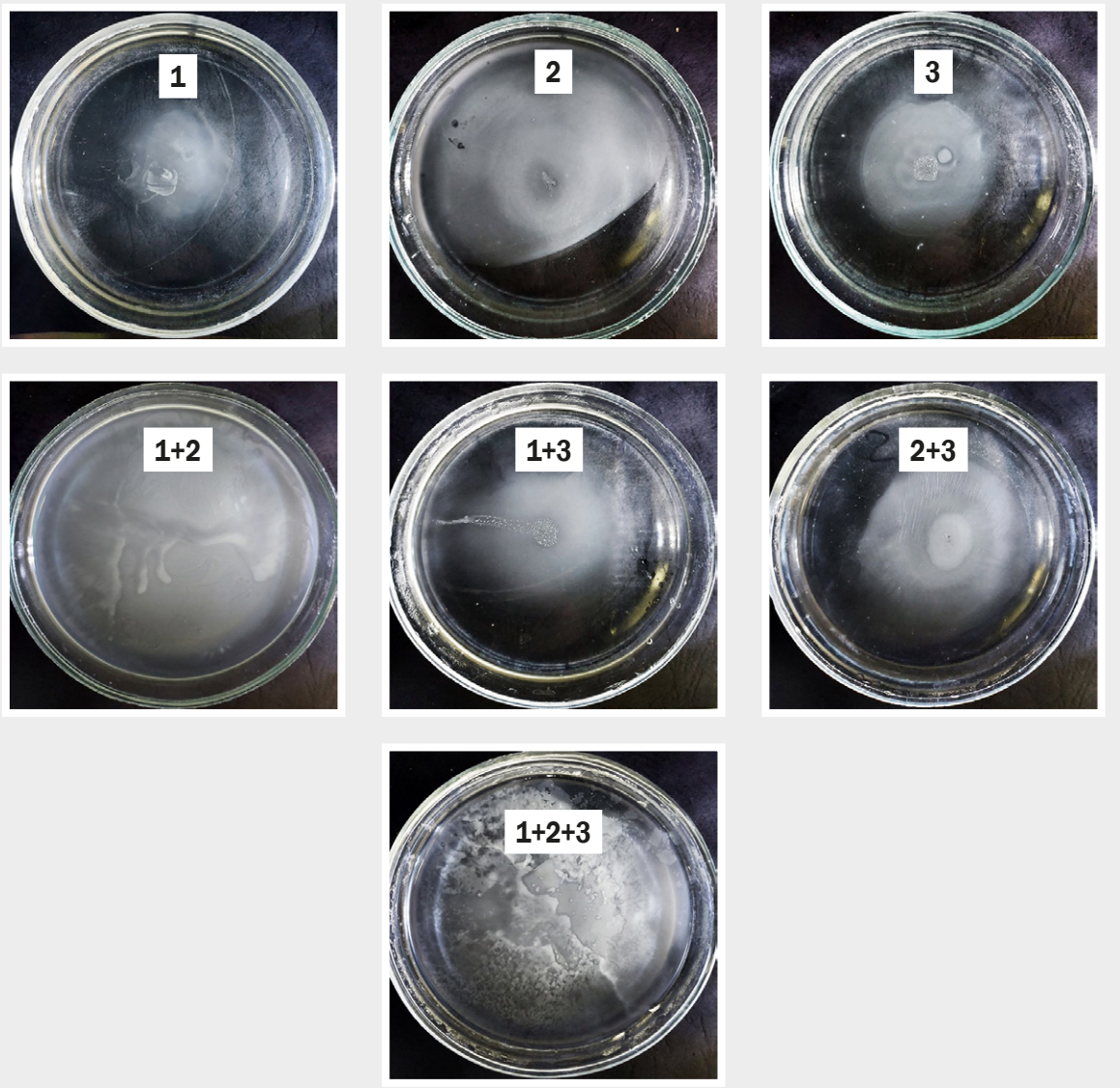


Fig. 3B. Representative photographs showing differences between the swimming halos formed by mono- and mixed cultures of the studied bacilli after 10 hours of cultivation. **1:** *B. clausii*; **2:** *B. coagulans*; **3:** *B. subtilis*.

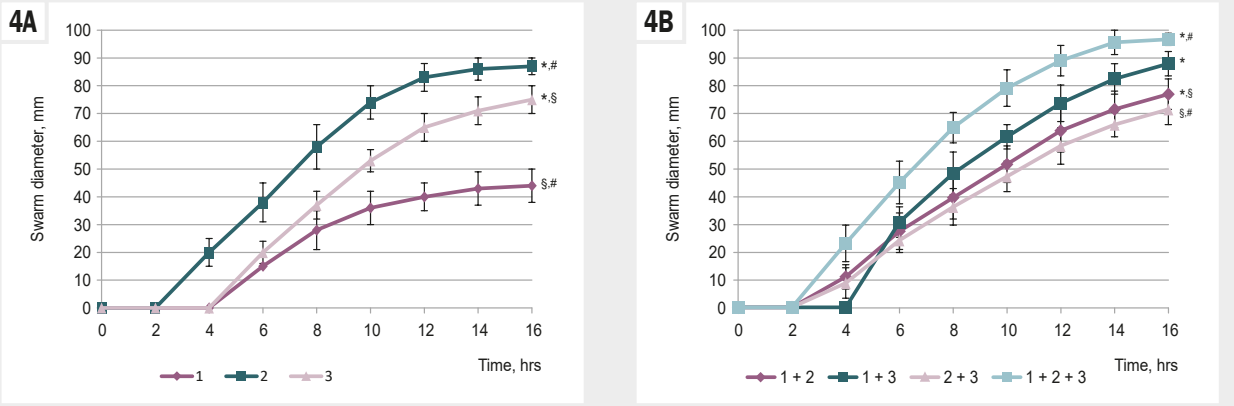


Fig. 4A, 4B. Swarming ability of *Bacillus* probiotic species under conditions of mono- (**A**) and co-cultivation (**B**).
***:** the differences are significant compared to 1; **§:** the differences are significant compared to 2; **#:** the differences are significant compared to 3, $p < 0.05$.

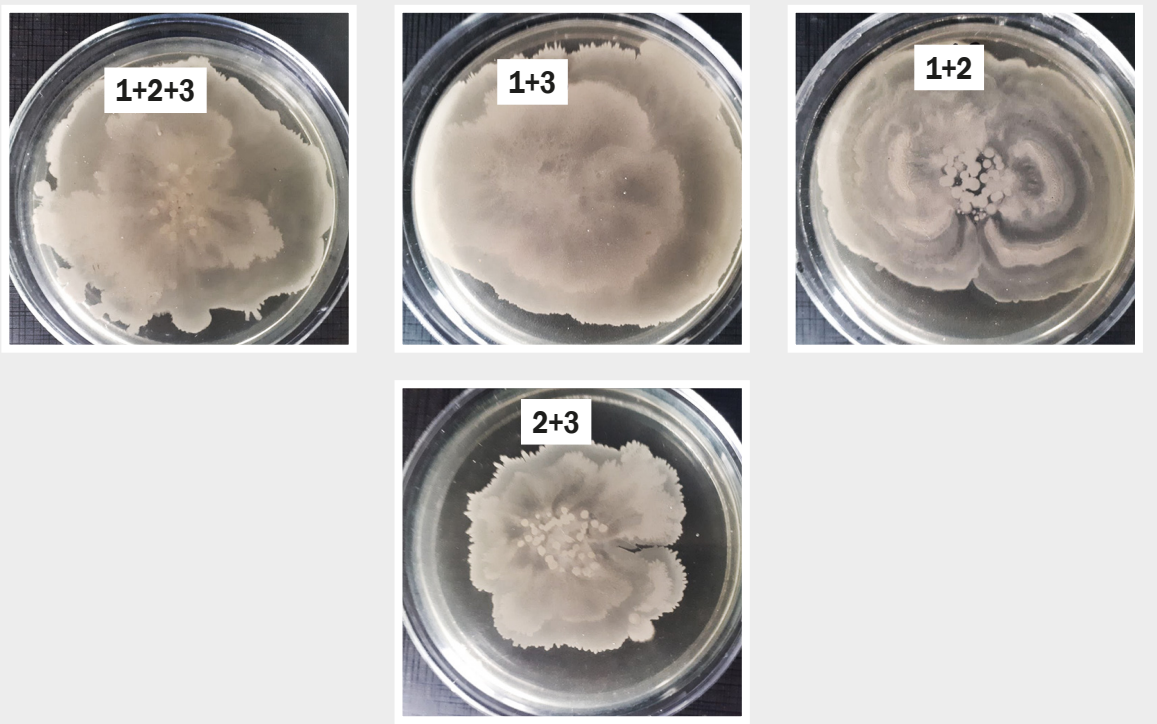


Fig. 4C. Representative photographs showing the differences between swarms formed by mixed cultures of bacilli. **1:** *B. clausii*; **2:** *B. coagulans*; **3:** *B. subtilis*.

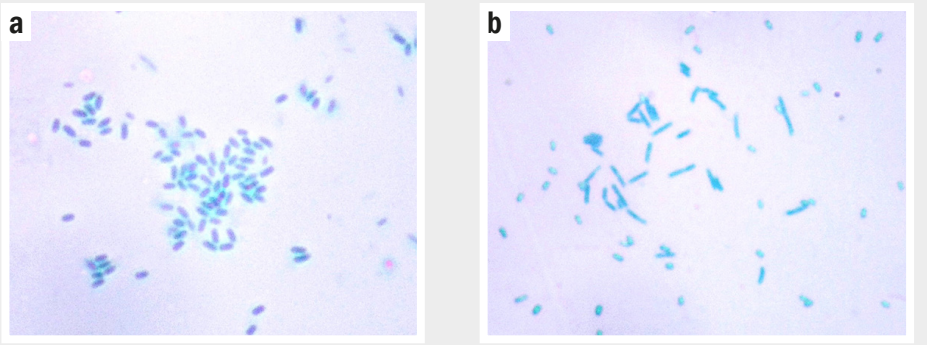
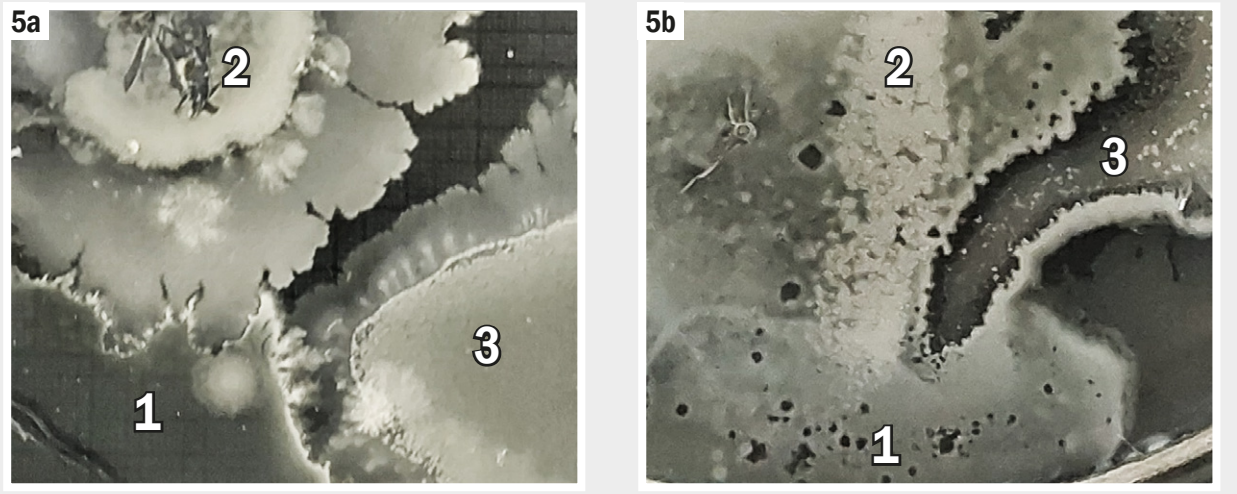


Fig. 4D. Representative micrographs showing differences between normal (**a**) and elongated swarm (**b**) cells. Normal cells were 2.0–2.5 × 1.5 μm in size; swarm cells had a size of 3.5–4.5 × 0.5–1.0 μm. Anjesky's staining technique.



1: *B. clausii*; **2:** *B. coagulans*; **3:** *B. subtilis*.

Fig. 5. Representative photographs to show the differences in approach phenotypes between swarms of probiotic bacillus species after 10 (**a**) and 24 (**b**) hours of cultivation.

to 2.5 ± 0.5 hours. The *B. clausii* + *B. subtilis* mixed culture was characterized by a longer lag period, 4.0 ± 0.2 hours. The triple mixed culture was in a lag period of 2.00 ± 0.25 hours. The average swarm expansion rates calculated for *B. clausii* + *B. coagulans; B. clausii* + *B. subtilis; B. coagulans* + *B. subtilis* and *B. clausii* + *B. coagulans* + *B. subtilis* mixed cultures were ~5.2 mm/h, ~6.7 mm/h, ~4.8 mm/h and ~6.3 mm/h, respectively. As can be seen from the data presented in *Fig. 4 B* and *C*, after 16 hours of cultivation, the swarm formed by *B. clausii* + *B. coagulans* mixed culture covered the plate surface by 60.5 ± 8.0 % (which was significantly more than the swarm formed by *B. clausii* culture, but significantly less than the swarm formed by *B. coagulans* culture). The swarm formed by *B. clausii* + *B. subtilis* mixed culture covered the plate surface by 79.0 ± 7.9 % (which was significantly more than the swarm formed by *B. clausii* culture but did not significantly differ from the swarm formed by *B. subtilis* culture alone). The swarm formed by *B. coagulans* + *B. subtilis* mixed culture covered the plate surface by 52.2 ± 7.4 %, which was significantly less than the swarms formed by each culture separately. The swarm formed by a mixture of three cultures covered the plate surface by 95.6 ± 4.3 %, which was is significantly more than the swarms formed by *B. clausii* and *B. subtilis* cultures separately, but did not differ from the swarm formed by *B. coagulans* culture alone (*Fig. 4A,B,C*).

We also examined approach phenotypes between swarms of *B. clausii*, *B. coagulans* and *B. subtilis.* At the meeting point of the studied swarms, we always observed a visible boundary (*Fig. 5a, b*). Between swarms of *B. subtilis* and *B. clausia* as well as between swarms of *B. subtilis* and *B. coagulans* were observed very striking and clear lines. They could be called "boundary" lines. Between swarms of *B. clausia* and *B. coagulans* we observed visible, but less bright lines. They could be called "intermediate" lines. We have not been able to detect mergers of swarms of different species.

Discussion

To expand the range, populate new ecological niches, adapt to adverse environmental factors, competitive or cooperative interaction with other species, bacteria use a number of well-regulated forms of population behavior [28,29].

Bacteria that have entered a common environment inevitably interact with each other. Such interaction leads to gradual adaptation to a complex life together [30]. Interactions between bacteria can change bacterial gene expression patterns and induce the secretion of various molecules that ensure survival in conditions of natural intraspecies and interspecies competition by suppressing or eliminating weak competing species or subpopulations [30,31]. The antagonistic activity of bacteria against closely related species or the entire genus is provided primarily by the production of bacteriocins and bacteriocin-like inhibitory substances (BLIS) [32,33]. They are united in a heterogeneous group of bioactive peptides and proteins synthesized on bacterial ribosomes or produced via non-ribosomal synthesis, with different biochemical properties, molecular weight, amino acid sequence and mechanism of action [32,34,35]. These antimicrobial peptides can be bacteriostatic or bactericidal not only against closely related, but also against a wide range of unrelated to producer groups of microorganisms, including those resistant to antibiotics, which makes them a promising alternative to antibiotics [8,34,35,36,37].

Since the production and secretion of bacteriocins require high metabolic costs, bacteria have developed regulatory mechanisms for their generation only upon necessity [38]. As long as there are no competitors in the environment, the production of bacteriocins remains at a low level or may be gradually lost under favorable laboratory conditions. Bacteriocin-producing bacteria can be induced to increase the production of bacteriocins by bacteriocin-inducing microorganisms. Both bacteria and fungi can act as inducers of bacteriocin formation. For example, co-cultivation of *Wickerhamomyces anomalus* Y-5 and *Lactiplantibacillus paraplantarum* RX-8 can enhance the production of plantaricin by the

latter [39]. Co-cultivation with pathogenic *Listeria monocytogenes* or *Salmonella enterica* results in increased production of the more mature and active nisin by *Lactococcus lactis* [22].

Probably, inhibition of the growth of indicator cultures under the influence of test cultures of bacilli, observed in the study described here, was due to the action of inhibitory compounds of the bacteriocin class or BLIS. We assume that under conditions of co-cultivation, some species of bacilli could play the role of inducers or enhancers of bacteriocin production by other species of bacilli. Therefore, the combination of probiotic *Bacillus* species can increase their overall inhibitory potential against pathobionts and pathogenic bacteria. This assumption remains to be confirmed in further studies.

The results of the study of the inhibitory activity of *B. clausii* and *B. coagulans* by two different methods were similar. The inhibitory activity of *B. subtilis* was stronger when using the agar block method. Obviously, the reasons for this difference must be sought in the features of research methods. In the agar block method, a more mature test culture with an agar substrate impregnated with the products of its metabolism is planted on a freshly sown indicator culture lawn. A spot-on-lawn assay fundamentally differs from the agar block method by the simultaneous inoculation of indicator and test cultures on a nutrient medium. This means that the growth phases of both cultures pass synchronously and the *B. subtilis* test culture does not have time to synthesize a sufficient amount of inhibitory compounds.

Swimming and swarming motility is an important survival strategy that allows bacteria to move across surfaces to nutrient-rich niches with optimal conditions for growth and reproduction, successfully colonize the mucosa, establish a population, and interact with target host cells [24,40]. Motile cells with an intact chemotaxis mechanism ensure early colonization. Subsequently, part of the motile cells differentiates into non-motile cells capable of forming the extracellular matrix. The formation of a multicellular bacterial community covered by a self-secreting matrix, the so-called biofilm, is necessary for stable long-term colonization [41].

Swimming motility involves the movement of individual cells in a three-dimensional fluid space due to the rotation of the flagella [28]. The ability to actively move varies greatly between strains [42]. Bacteria in a common environment can affect each other's motility. For example, the swimming motility of *Methylobacterium* sp. ME121 was increased when co-cultivated with an immobile *Kaistia* sp. 32K, which produced a polysaccharide acceleration factor [21]. The motility of *Salmonella enterica serovar Typhimurium* was either reduced or lost due to exposure to acidic metabolites of *Lactococcus lactis* subsp. lactis during their co-cultivation [43]. *B. clausii, B. coagulans* and *B. subtilis* are able to swim in a liquid environment due to peritrichially located flagella [26,44]. This study revealed the mutual influence of some studied species of bacilli on each other's swimming activity. Comparison of the growth rates of swimming halos formed by the *B. clausii* + *B. subtilis* mixed culture with halos formed by each culture separately led to the conclusion that one or both cultures stimulated the swimming motility of the other. The same could not be said about the *B. clausii* + *B. coagulans* or *B. coagulans* + *B. subtilis* mixed cultures. It was found out that the *B. clausii* + *B. coagulans* + *B. subtilis* mixed culture was able to cover a significantly larger area of the swimming plate than any of the cultures for the same cultivation time.

Swarming motility is genetically distinct from swimming [44]. Swarming is an example of a multicellular coordinated population behavior, collective migration of bacteria consisting of the rapid movement of groups of flagellar cells in a thin liquid film on a semi-solid surface [28,29]. Contact with a harder surface slows the movement of flagella, cells undergo differentiation, becoming elongated serpentine or rod-like, the number of flagella increases [28,43]. In some species, the types of flagella used for swimming differ from those used for swarming motility [28]. Successive phases of swarming have certain spatio-temporal regularities. During several hours of the initial lag phase, the swarm does not migrate outward; there is a significant increase in cell density, the synthesis of additional flagella and the production of surfactin. Then there is a sharp transition to the phase of exponential swarm expansion, during which a collective movement in the form of cell rafts is observed inside the swarm [29]. Expansion rates can reach 5–36 mm/h and the swarm can cover the entire agar plate within hours or days [28]. The results of this study have demonstrated the mutual influence of some studied species of bacilli on each other's swarming ability. This is evidenced by the fact that the swarming potential of the *B. coagulans* + *B. subtilis* mixed culture was significantly lower, and the swarming potential of the *B. clausii* + *B. subtilis* and the triple mixed cultures was significantly higher than theoretically expected.

The relatively high rate of swimming halos growth and the rapid swarm expansion of the *B. clausii* + *B. coagulans* + *B. subtilis* mixed culture may indicate its high capacity for early colonization. Further *in vitro* and *in vivo* studies using animal models are necessary for the final determination of the colonization potential of this triple mixed culture.

When bacilli swarm in a common environment, a differentiated attitude of neighbors towards each other is revealed [27]. They are able to distinguish kin from nonkin depending on phylogenetic kinship. This phenomenon was called "kin discrimination". Genetically identical swarms show the ability to merge, while swarms composed of different strains form a visible boundary between themselves and do not merge. Belonging to different species explains the fact that the studied bacilli did not show the ability to merge swarms, but, on the contrary, their swarms at the point of contact formed visible "boundary" or "intermediate" lines, demonstrating the ability to identify nonself. The formation of a bright boundary line at the point of contact of two swarms indicates an antagonistic relationship between species. Antagonism between swarms may extend to other multicellular contexts [27]. Thus, the subsequent study of the mutual influence of the studied cultures in the process of biofilm formation is of scientific interest.

Conclusions

1. The obtained results indicate the ability of the probiotic species of bacilli: *B. clausii*, *B. coagulans* and *B. subtilis* to mutually influence growth, swimming and swarm motility.

2. The revealed moderate and weak inhibitory activity of some species of bacilli against others allows us to assume

that the overall inhibitory potential of mixed cultures of bacilli against pathobionts and pathogenic bacteria is higher than that of monocultures due to the mutual induction of the production of inhibitory compounds by bacilli. This assumption is subject to further confirmation.

3. The obtained relatively high rate of swimming halos growth and the rapid swarm expansion of the *B. clausii* + *B. coagulans* + *B. subtilis* mixed culture testify to its high ability for early colonization. Further *in vitro* and *in vivo* studies using animal models are necessary for the final determination of the colonization potential of this triple mixed culture.

Prospects for further research: obtained results will be used in the development of new probiotic preparations based on a combination of probiotic bacilli.

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