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Bioengineering of probiotic-loaded hydrogel films with high antimicrobial activity

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The rise of antimicrobial resistance complicates the management of infected wounds, necessitating novel therapeutic strategies. Probiotic-based therapies offer a promising alternative, but their efficacy depends on delivering a high concentration of viable, active microorganisms to the wound site. Alginate hydrogels are excellent carriers, but freshly prepared probiotic-loaded films often lack immediate therapeutic activity. This study aimed to investigate the effect of a post-immobilization cultivation period on the viability and antagonistic activity of *Bifidobacterium bifidum* LVA-3 and *Lactobacillus bulgaricus* 1Z 03501 immobilized in calcium alginate films. The central hypothesis was that this cultivation step would function as an in-situ bio-activation process, enhancing the films' therapeutic potential.
Methods. *B. bifidum* LVA-3 and *L. bulgaricus* 1Z 03501 were immobilized in calcium alginate films. The films were then cultivated in a nutrient medium for 2, 4, or 6 days at 37 °C. Viable cell counts were determined by plate counting after film dissolution. Antagonistic activity was assessed using an agar overlay diffusion method against pathogenic test strains (*Staphylococcus aureus* 209, *Pseudomonas aeruginosa* 9027, and *Escherichia coli* B), measuring the diameter of inhibition zones. The experimental data revealed that uncultivated films (Day 0) showed no antagonistic activity. Post-immobilization cultivation led to a significant increase in viable cell counts for both strains, with populations rising by over 100- to 500-fold within 2 days to therapeutically relevant levels (>10^{10} CFU/mL). *B. bifidum* LVA-3 showed rapid growth peaking at day 2, while *L. bulgaricus* 1Z 03501 maintained a high, stable population through day 6. This increased cell density directly correlated with the emergence of potent antagonistic activity against all three pathogenic strains. In summary, it can be concluded that a post-immobilization cultivation step is a critical bio-activation process that transforms probiotic-loaded alginate films from passive carriers into functionally potent biomaterials. This strategy effectively increases probiotic viability to therapeutic concentrations and enables the *in-situ* production of antimicrobial compounds. This two-step approach of immobilization followed by cultivation presents a novel method for developing high-efficacy probiotic formulations for applications such as bioactive wound dressings.

Key words*: probiotic; alginate hydrogel; immobilization; in-situ cultivation; antagonistic activity*

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Introduction

The management of infected wounds is a significant clinical challenge, further complicated by the global rise of antimicrobial resistance (AMR) (Aslam et al., 2018). Chronic wounds are frequently colonized by pathogens such as *Staphylococcus aureus* (Almuhayawi et al., 2023), *Pseudomonas aeruginosa* (Phan et al., 2023), and *Escherichia coli* (Wang et al., 2025), which form resilient biofilms, which are highly resistant to both the host immune response and conventional antibiotic therapies (Landa et al., 2024; Vestweber et al., 2024). These bacterial species are widely used as test microorganisms in the development and evaluation of innovative antibacterial wound dressings (Alberts et al., 2025; Karachevtsev et al., 2025).

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The diminishing efficacy of existing antibiotics against multidrug-resistant strains necessitates a shift towards innovative, non-antibiotic alternatives (Ilyas et al., 2024; Byk et al., 2024; Singh et al., 2024). An ideal wound therapy should not only exert antimicrobial activity but also modulate the chronic inflammatory response to promote the physiological healing process (Jafari et al., 2024; Zhang et al., 2025; Kong et al., 2022).

In this context, probiotic-based interventions have emerged as a promising strategy (Bădăluță et al., 2024; Gul et al., 2024). Probiotics, defined as live microorganisms that confer a health benefit when administered in adequate amounts, can inhibit wound pathogens via several mechanisms (Abdul et al., 2025; Safronova et al., 2024). These include competition for nutrients and adhesion sites, disruption of pathogen communication systems, and the production of antimicrobial substances like organic acids and bacteriocins (Babenko et al., 2022). Furthermore, probiotics possess immunomodulatory properties capable of resolving the persistent inflammation that stalls healing (Mazziotta et al., 2023). Among the most studied genera for these applications are *Lactobacillus* and *Bifidobacterium* (Picó-Monllor et al., 2023; Xiao et al., 2021; Dueñas et al., 2022).

Successful topical delivery of these biotherapeutics relies on an effective carrier system, with alginate hydrogels being a leading choice (Diep et al., 2024; Sun et al., 2023; Trufanov et al., 2025). Alginate hydrogels are biocompatible, maintain a moist healing environment by absorbing wound exudate, and facilitate the sustained release of therapeutic agents (Lou et al., 2025; Abourehab et al., 2022). However, a key limitation is that freshly prepared films with immobilized probiotics often exhibit insufficient antagonistic activity for a therapeutic effect. This has led to the development of a novel strategy: utilizing the probiotic-loaded hydrogel as an in-situ bioreactor (Huang et al., 2023).

Our central hypothesis is that a post-immobilization cultivation step can function as a bio-activation process. During this incubation, immobilized probiotics can proliferate to a therapeutically relevant density and secrete a concentrated mixture of antimicrobial postbiotics within the alginate matrix. This approach aims to transform the hydrogel from a passive carrier into a functionally potent biomaterial. Therefore, the present study was designed to investigate the effect of a post-immobilization cultivation period on the viability and antagonistic activity of *Bifidobacterium bifidum* LVA-3 and *Lactobacillus bulgaricus* 1Z 03501 immobilized in calcium alginate films.

Materials and Methods

Reagents and Culture Media. Nutrient agar (Farmaktiv, Ukraine), nutrient broth (Conda, Spain), and semi-solid Blaurock medium were used for microbial cultivation. Key reagents included lactose, peptone, and calcium chloride (Khimlaborreaktiv, Ukraine); NaCl; cysteine; dry agar-agar (Conda, Spain); sodium alginate (Farma Sino, China); and trisodium citrate (Khimlaborreaktiv, Ukraine).

Blaurock medium was prepared from fresh beef liver (1 kg), which was cut into 1.5–2 cm pieces, soaked in distilled water, and kept at 4 °C for 10–12 hours. The liver was then boiled for 1 hour, and the resulting broth was filtered through gauze to a final volume of 1.0 L. To this broth, lactose (10 g), peptone (10 g), and NaCl (5 g) were added. The mixture was boiled for an additional 15 minutes, supplemented with cysteine (0.1 g), cooled to 25 °C, and adjusted to pH 7.2. The solution was filtered through filter paper, mixed with dry agar-agar (0.75 g), and sterilized in a steam autoclave (VK-75, Ukraine) at 0.8 atm for 40 minutes.

Microbial Strains and Culture Conditions. All microbial strains were obtained from the collection of the Cryomicrobiology Department at the Low Temperature Bank of Biological Objects, Institute for Problems of Cryobiology and Cryomedicine, NAS of Ukraine. Stock cultures were stored in NUNC cryovials (Thermo Fisher Scientific, USA) in appropriate cryoprotective media at −196 °C in liquid nitrogen.

The probiotic strains *Bifidobacterium bifidum* LVA-3 and *Lactobacillus bulgaricus* 1Z 03501 were stored frozen in Blaurock medium. For activation, cryovials were thawed in a 40 °C water bath, and the contents were transferred into semi-solid Blaurock medium and incubated at 37 °C for 48 hours to obtain starter cultures.

The conditionally pathogenic test strains *Escherichia coli* B, *Staphylococcus aureus* 209, and *Pseudomonas aeruginosa* 9027 were used to evaluate antagonistic activity. These strains were stored frozen in nutrient broth. For use, they were thawed in a 40 °C water bath, inoculated onto slanted nutrient agar, and subcultured weekly.

Preparation of Probiotic-Loaded Hydrogel Films. A 0.4 mol/L aqueous solution of anhydrous calcium chloride was used as the crosslinking agent. A 40 g/L solution of trisodium citrate was prepared in distilled water with mechanical stirring to be used for dissolving the hydrogel films. A 30 g/L solution of sodium alginate was prepared by mixing the powder with distilled water and incubating at 37 °C for 18–20 hours under mechanical stirring. The $CaCl_2$ and trisodium citrate solutions were sterilized by autoclaving (0.8 atm, 40 min), while the sodium alginate solution was sterilized by tyndallization (100 °C for 1 hour on three consecutive days).

Starter cultures of *B. bifidum* LVA-3 and *L. bulgaricus* 1Z 03501 were diluted 100-fold in sterile distilled water. The diluted suspensions were then mixed with the sterile 30 g/L sodium alginate solution in a 1:2 volume ratio (10 mL culture to 5 mL alginate solution). Aliquots (0.4 mL) of this mixture were dispensed into the wells of a 24-well plate and crosslinked by treating with an aerosol of 0.4 mol/L calcium chloride solution for 15 minutes at room temperature. To remove unreacted calcium ions, the resulting films were washed three times by immersion in a tenfold volume of sterile distilled water at 25 °C for 20 minutes per wash.

Experimental Design. Following immobilization and washing, three films were placed into penicillin vials containing 10 mL of sterile Blaurock medium. For each probiotic strain, four experimental groups were established (n=10 vials per group).

Group 1. Stored for 6 days at −24 °C (control).

Group 2. Incubated at 37 °C for 2 days, then stored at −24 °C for 4 days.

Group 3. Incubated at 37 °C for 4 days, then stored at −24 °C for 2 days.

Group 4. Incubated at 37 °C for 6 days.

Evaluation of Viability and Antagonistic Activity. All analyses were performed on day 6 of the experiment. Antagonistic activity was assessed using an agar overlay diffusion method. Molten nutrient agar was poured into Petri dishes and inoculated with a suspension of a test strain (*E. coli* B, *S. aureus* 209, or *P. aeruginosa* 9027). After surface drying for 30 minutes, four alginate films from a given experimental group were placed on the agar surface. The plates were incubated at 37 °C for 12 hours under aerobic conditions. Antagonistic activity was quantified by measuring the diameter of the inhibition zones around the films after 6 and 12 hours of incubation.

To determine viable cell concentration, one film was immersed in 20 mL of sterile 40 g/L trisodium citrate solution and incubated at 25 °C for 60 minutes with periodic shaking until complete dissolution. The resulting suspension was serially diluted, plated in Blaurock medium, and incubated at 37 °C for 48 hours. Viable cells were quantified by counting the resulting macrocolonies.

Statistical Analysis. All experiments were performed in triplicate unless otherwise specified. Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using Microsoft Excel and GraphPad Prism software. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to assess differences between groups. A p-value < 0.05 was considered statistically significant.

Results

Growth and Viability of Immobilized Probiotics During In-Situ Cultivation. The cultivation of probiotic cultures within the calcium alginate films demonstrated that the hydrogel matrix provides a suitable environment for bacterial proliferation. In the semi-solid Blaurock medium, macrocolonies of *B. bifidum* LVA-3 and *L. bulgaricus* 1Z 03501 exhibited their characteristic morphologies, appearing as "comet-like" and "ball-like" structures, respectively.

Quantitative analysis revealed a dramatic increase in the viable cell counts for both strains following cultivation (Figure 1). The initial cell density in the films immediately after immobilization (Day 0) was approximately 8.3±0.4 log CFU/mL for *B. bifidum* LVA-3 and 9.6±0.5 log CFU/mL for *L. bulgaricus* 1Z 03501. These results underscore that the immobilization process, which included a 100-fold dilution, results in a relatively low starting probiotic load.

The two strains exhibited distinct growth kinetics within the films.

B. bifidum LVA-3 demonstrated rapid, exponential growth, with its viable cell count increasing by approximately 2.7 log units (a ~500-fold increase) to a peak of 11.0±0.6 log CFU/mL by day 2. After this peak, the population entered a decline phase, decreasing to 9.9±0.5 log CFU/mL by day 6. This pattern is characteristic of a classic batch culture growth curve. In contrast, *L. bulgaricus* 1Z 03501 showed a more sustained growth pattern. Its population increased by over 2.0 log units (a >100-fold increase) by day 2, reaching 11.6±0.3 log CFU/mL, and this high cell density was maintained in a stable stationary phase through day 6. For both strains, all values for days 2, 4, and 6 were significantly different ($p < 0.05$) from the day 0 control.

Emergence and Characterization of Antagonistic Activity Post-Cultivation. A central finding of this study is that the control films (0 days of cultivation) did not produce any detectable inhibition zones (0 mm) against the three pathogenic test strains (Figures 2-4).This initial lack of functional activity suggests that the cell concentration in the uncultivated films was below the critical threshold necessary to produce sufficient quantities of antimicrobial substances. Furthermore, it is likely that any previously synthesized antagonistic compounds were lost via diffusion during the ionotropic gelation process.

Antagonistic activity emerged and was enhanced in direct correlation with cultivation time and the corresponding increase in cell population (Figure 2, 3, 4). Comparing the two probiotic strains,

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B. bifidum LVA-3 generally exhibited stronger antagonistic activity than *L. bulgaricus* 1Z 03501. The inhibition zones produced by *B. bifidum* LVA-3 against *S. aureus* 209 and *P. aeruginosa* 9027 were consistently larger (by 15-50%). For instance, after 6 days of cultivation, *B. bifidum* LVA-3 produced a 15 mm zone against *S. aureus* 209, while *L. bulgaricus* 1Z 03501 produced only a 10 mm zone (Figure 5, 6). Both strains showed comparable, though more modest, activity against *E. coli* B.

When comparing pathogen sensitivity, *P. aeruginosa* 9027 appeared to be the most susceptible to the probiotics, followed by *S. aureus* 209 and then *E. coli* B. A further observation was that inhibition zones decreased in size as the assay incubation period was extended from 6 to 12 hours (Figure 2).

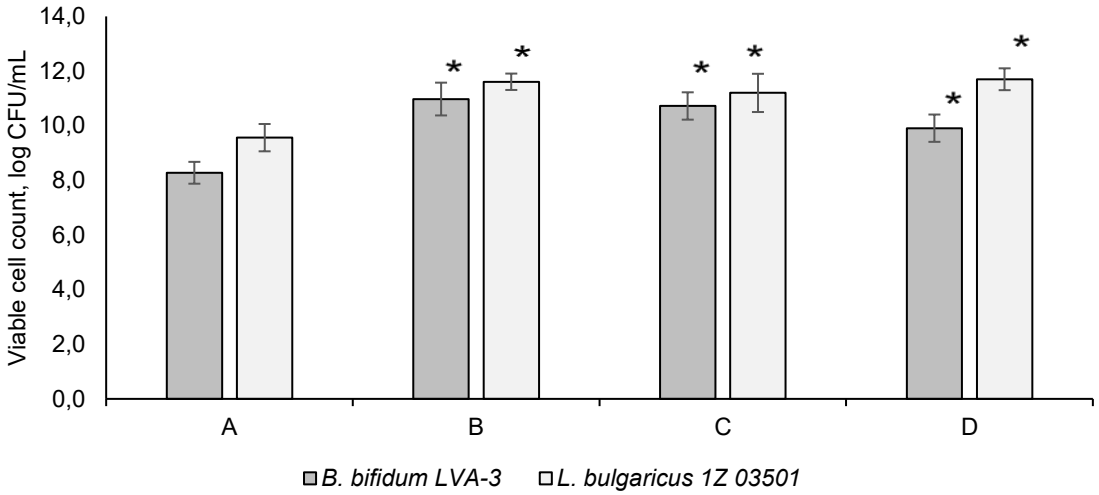

Fig. 1. Viable cell counts of *B. bifidum* LVA-3 and *L. bulgaricus* 1Z 03501 (log CFU/mL) in alginate films: A – control (without cultivation), B – after cultivation for 2 days, C – after cultivation for 4 days, D – after cultivation for 6 days. *Data are presented as mean ± SD from triplicate experiments. All values for days 2, 4, and 6 are significantly different ($p < 0.05$) from the day 0 control for each respective strain.*

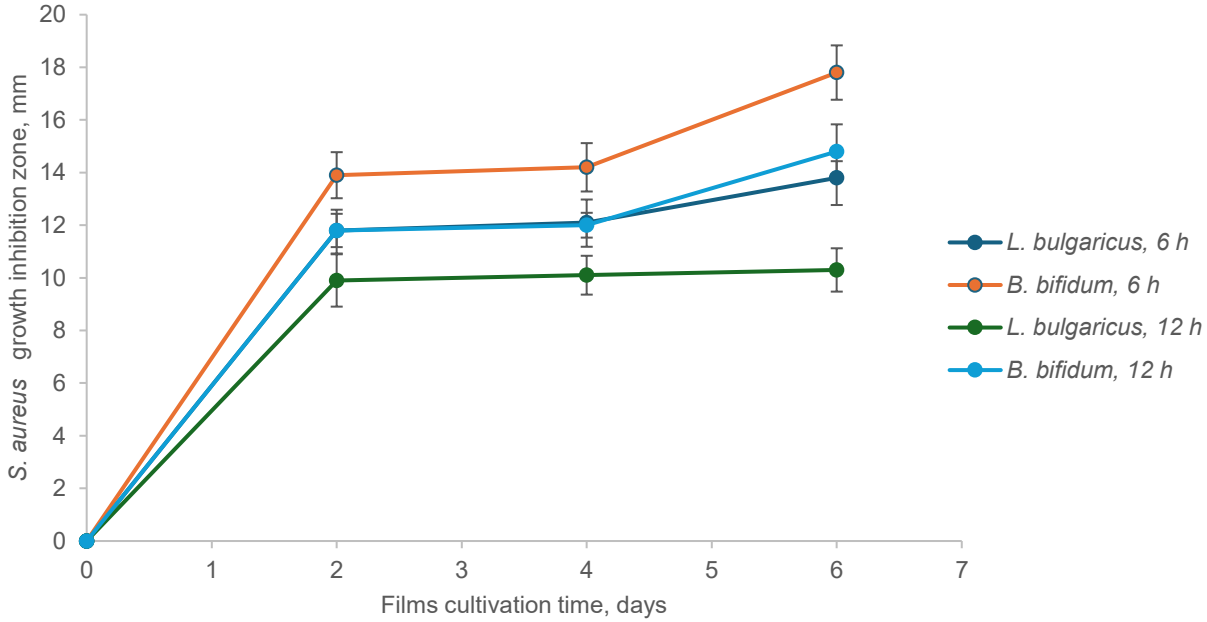

Fig. 2. Antagonistic activity (inhibition zone diameter, mm) of cultivated probiotic films against *S. aureus* 209 after 2-6 days of incubation.

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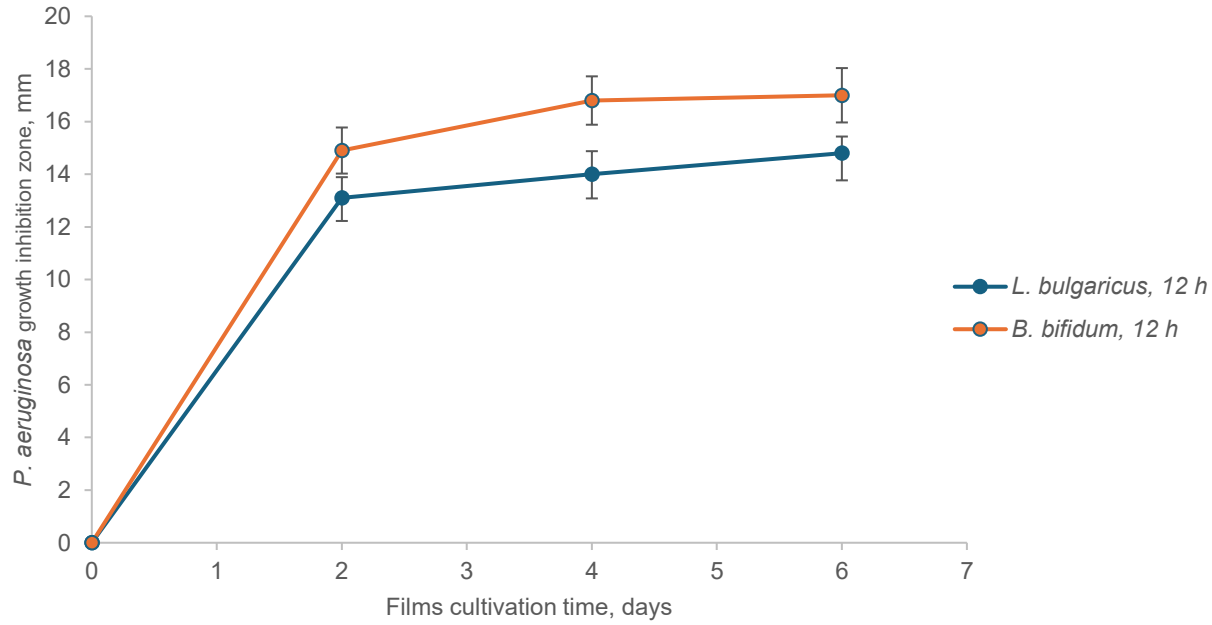

Fig. 3. Antagonistic activity (inhibition zone diameter, mm) of cultivated probiotic films against *P. aeruginosa* 9027 after 2-6 days of incubation.

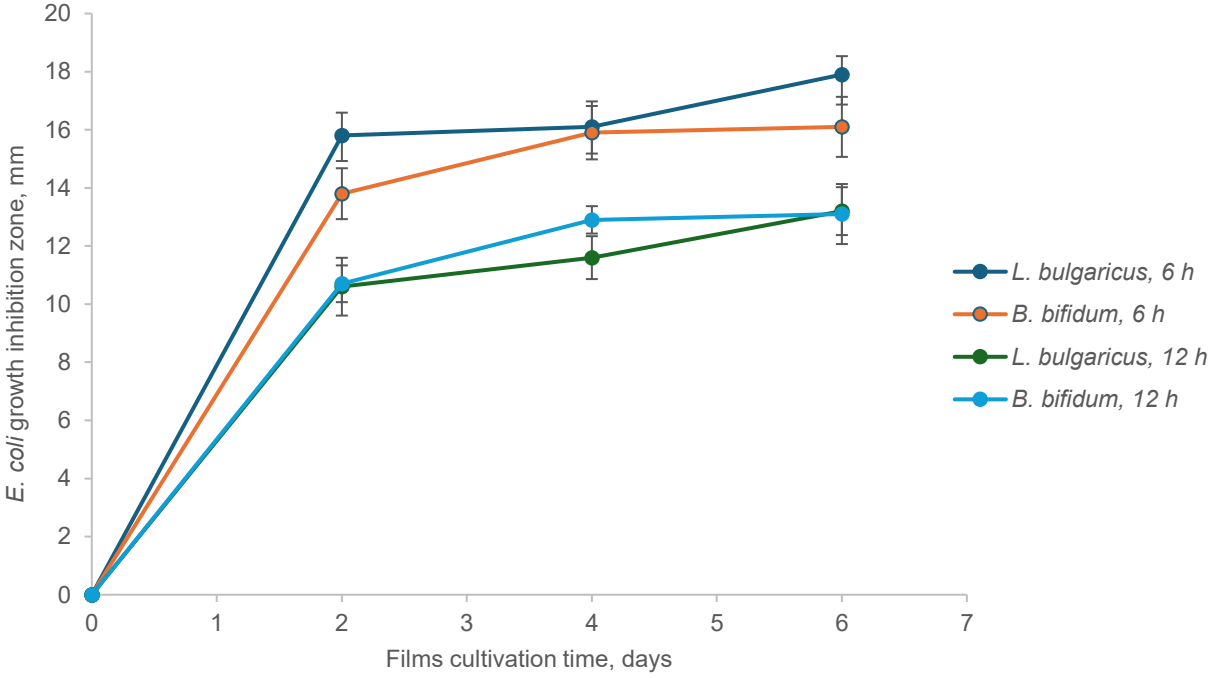

Fig. 4. Antagonistic activity (inhibition zone diameter, mm) of cultivated probiotic films against *E. coli* B after 2-6 days of incubation.

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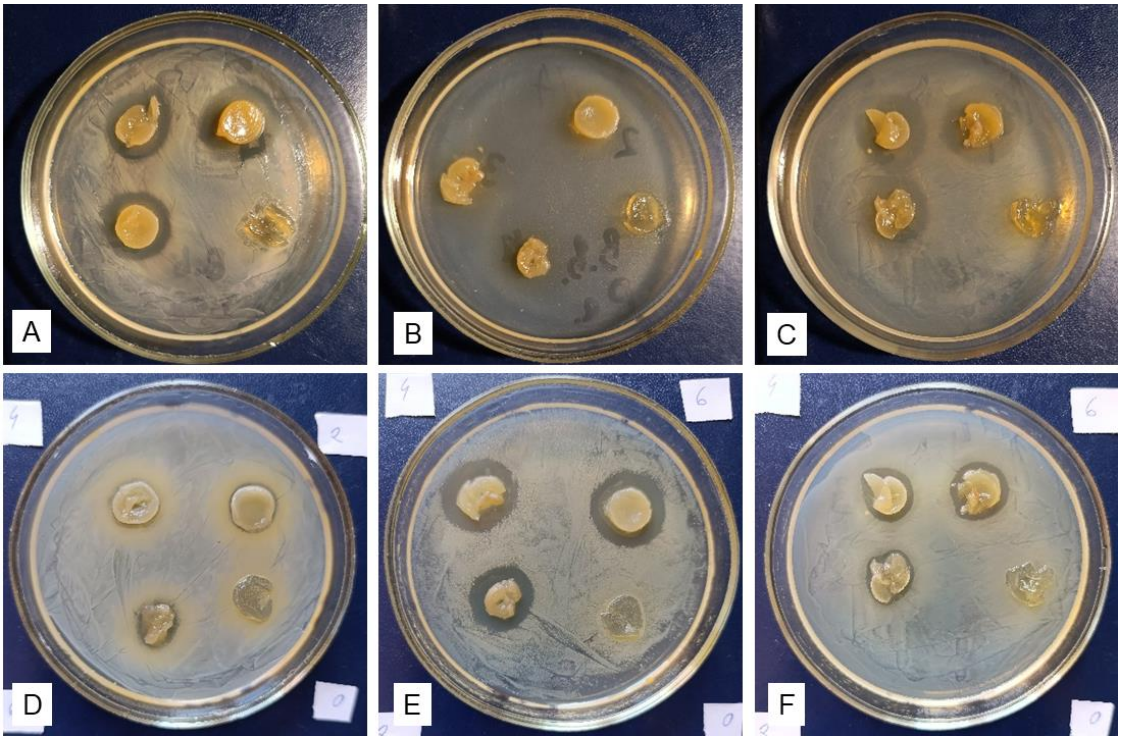

Fig. 5. Effect of cultivation time on the growth of test microorganisms and the antagonistic activity of *B. bifidum* LVA-3 immobilized in alginate films. A — *S. aureus* 209, 6 h; B — *P. aeruginosa* 9027, 6 h; C — *E. coli* B, 6 h; D — *S. aureus* 209, 12 h; E — *P. aeruginosa* 9027, 12 h; F — *E. coli* B, 12 h.

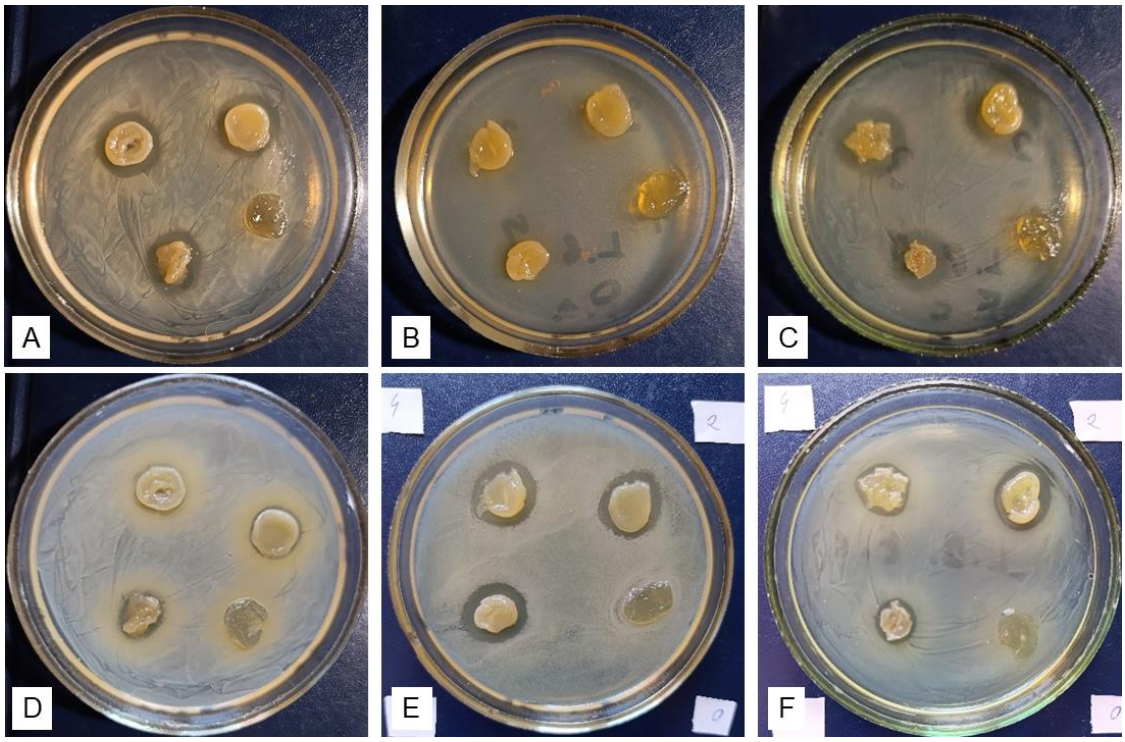

Fig. 6. Effect of cultivation time on the growth of test microorganisms and the antagonistic activity of *L. bulgaricus* 1Z 03501 immobilized in alginate films. A — *S. aureus* 209, 6 h; B — *P. aeruginosa* 9027, 6 h; C — *E. coli* B, 6 h; D — *S. aureus* 209, 12 h; E — *P. aeruginosa* 9027, 12 h; F — *E. coli* B, 12 h.

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Discussion

The experimental design was centered on the central idea of in-situ bio-activation, in which the probiotic-loaded hydrogel serves as a small-scale bioreactor capable of producing both a therapeutically relevant cell density and an effective level of antibacterial metabolites. The choice of probiotic genera, *Lactobacillus* and *Bifidobacterium*, was based on their established roles in pathogen inhibition and immunomodulation, which are critical for resolving the persistent inflammation characteristic of chronic wounds (Picó-Monllor et al., 2023; Xiao et al., 2021; Dueñas et al., 2022). The use of specific, cryopreserved strains from the institutional collection (*Bifidobacterium bifidum* LVA-3 and *Lactobacillus bulgaricus* 1Z 03501) ensures the reproducibility of the experiments. Similarly, the selection of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* as test pathogens ensures high clinical relevance, as they are the predominant isolates in infected wounds (Diep et al., 2024; Sun et al., 2023; Trufanov et al., 2025). The differential susceptibility observed, with *P. aeruginosa* being the most sensitive, provides valuable insight into the potential spectrum of activity of the developed biomaterials.

Methodologically, the choice of a calcium alginate hydrogel is justified by its excellent biocompatibility, biodegradability, and ability to provide a protective, moisture-retentive environment for the encapsulated probiotics and its suitability for potential wound dressing applications (Lou et al., 2025; Abourehab et al., 2022).

This study confirms that immobilization in calcium alginate does not impede cell division and population growth; rather, the matrix acts as a scaffold supporting robust proliferation. The cultivation of bacterial cultures in an immobilized state within a calcium alginate hydrogel was demonstrated, with kinetic parameters similar to those of free-cell cultures. This immobilized cultivation offers advantages, including preventing cell sedimentation, thus eliminating the need for agitation, and providing a protective effect from the hydrogel polymers, analogous to natural biofilms. It is well known that alginate is a natural component of bacterial biofilms, which contributes to this protective function (Dai et al., 2002; Kundukad et al., 2025).

The observed increase in cell densities to levels commonly associated with therapeutic efficacy ($>10^9$–10^{10} CFU/mL) confirms the success of the in-situ cultivation approach (Gul et al., 2024). These findings are consistent with other studies that have reported successful cultivation of immobilized lactobacilli and bifidobacteria in various matrices (Huang et al., 2023). The distinct growth kinetics of the two strains have practical implications: a 2-day cultivation of *B. bifidum* LVA-3 is optimal for applications requiring a rapid burst of high cell density, whereas *L. bulgaricus* 1Z 03501 is more suitable for sustained high viability.

The emergence of antagonistic activity was directly dependent on reaching a "functional activation threshold" of cell density through cultivation (Goo et al., 2024; Thiemicke et al., 2023). The cultivation process allowed the probiotic population to increase to a density where its collective metabolic output of antimicrobial compounds—such as organic acids and bacteriocins—was sufficient to create a measurable zone of inhibition. The alginate hydrogel structure facilitates this process, as its pore size retains the bacterial cells while allowing for the outward diffusion of these smaller antimicrobial molecules to act on surrounding pathogens (Eiselt et al., 2000; Tang et al., 2021).

The decrease in inhibition zones over time suggests the primary mechanism of action is likely bacteriostatic (inhibiting growth) rather than bactericidal (killing cells). This finding is consistent with some recent studies (Ji et al., 2025) but contradicts others (Tang et al., 2021). The degradation of antimicrobial compounds over time provides a plausible explanation. Probiotics often produce proteinaceous bacteriocins as their primary antimicrobial agents (V et al., 2021; Ismael et al., 2024). Since pathogens like *S. aureus* 209 and *P. aeruginosa* 9027 secrete peptid-degrading proteases (Li et al., 2025; Simons et al., 2020; Kessler et al., 2024; Molujin et al., 2022), it is likely that the initial inhibition is caused by a burst of bacteriocins, which are subsequently degraded by pathogen-produced proteases, allowing the pathogens to recover and resume growth. This reveals a dynamic interplay between the probiotic and pathogen on the agar plate.

A potential limitation of this study is the use of a beef liver-based medium. While classic for cultivating lactic acid bacteria (Yakovychuk et al., 2017; Sirchak et al., 2022), it presents a risk of antibiotic residues and batch-to-batch variability for modern applications (Adegbeye et al., 2024; Taha et al., 2024). Future work should transition to a defined, plant-based, or synthetic medium to ensure consistency and safety.

Conclusions

The findings of this study demonstrate that a post-immobilization cultivation step is a highly effective strategy for enhancing both the viability and the functional antagonistic properties of *B. bifidum* LVA-3 and Lactobacillus bulgaricus 1Z 03501 encapsulated in calcium alginate films. Immobilization in calcium alginate supports robust proliferation of both probiotic strains, with cell densities increasing by over 100- to 500-fold within 2 days of cultivation and reaching therapeutically relevant levels. The two strains exhibited different

growth kinetics, with *B. bifidum* LVA-3 showing a rapid peak followed by a decline, and *L. bulgaricus* 1Z 03501 maintaining a stable, high-density plateau, offering options for tailoring formulations to specific applications.

The emergence of antagonistic activity in the films was directly dependent on this cultivation-driven increase in cell density, which facilitated the biosynthesis and accumulation of antimicrobial compounds. Non-cultivated films were functionally inert, whereas cultivated films exhibited potent, strain-dependent inhibition of pathogenic bacteria.

This research establishes post-immobilization cultivation as a critical bio-activation process. It transforms the alginate films from simple, passive protective carriers into potent, high-density functional biomaterials. This two-step strategy—immobilization followed by activation—presents a novel approach for the production of next-generation probiotic formulations. This method could be applied to develop more effective products for a range of uses, including bioactive wound dressings and probiotic therapies, by ensuring that a high and functionally active dose of probiotics is delivered to the target site.

Future research should focus on the molecular identification of the antimicrobial compounds produced, testing the performance of these activated films in simulated infected wound models, and optimizing co-culture conditions to develop synergistic multi-strain formulations.

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Біоінженерія пробіотичних гідрогелевих плівок з високою антимікробною активністю

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Зростання антимікробної резистентності значно ускладнює лікування інфікованих ран, що зумовлює необхідність пошуку нових терапевтичних підходів. Одним із перспективних напрямів є застосування пробіотиків, ефективність яких залежить від доставки високої концентрації життєздатних мікроорганізмів безпосередньо в зону ураження. Альгінатні гідрогелі є ефективними носіями, проте свіжовиготовлені пробіотичні плівки часто не забезпечують миттєвої антимікробної дії. Метою роботи було дослідити вплив періоду культивування після іммобілізації на життєздатність та антагоністичну активність штамів *Bifidobacterium bifidum* LVA-3 та *Lactobacillus bulgaricus* 1Z 03501, іммобілізованих в альгінатно-кальцієвих плівках. Гіпотеза полягала в тому, що цей етап культивування слугує *in-situ* біоактивацією, яка підвищує терапевтичну ефективність плівок. Пробіотичні штами іммобілізували в альгінатно-кальцієвих плівках і культивували у поживному середовищі протягом 2, 4 або 6 діб при 37 °C. Життєздатність визначали методом висіву після

розчинення плівок. Антагоністичну активність оцінювали методом дифузії в агарі щодо патогенних тест-штамів (*Staphylococcus aureus* 209, *Pseudomonas aeruginosa* 9027 та *Escherichia coli* B) за діаметром зон інгібування. Результати проведеного дослідження показали, що плівки без культивування (0-й день) не мали антагоністичної активності. Культивування після іммобілізації сприяло значному підвищенню життєздатності обох штамів — зростання чисельності клітин у 100–500 разів уже через 2 доби до рівнів, достатніх для терапевтичного ефекту (>10^{10} КУО/мл). *B. bifidum* LVA-3 характеризувався швидким ростом з піком на 2-й день, тоді як *L. bulgaricus* 1Z 03501 підтримував стабільну високу чисельність до 6-го дня. Зростання щільності клітин напряму корелювало з посиленням антагоністичної активності щодо всіх патогенів. Підсумовуючи можна зазначити, що культивування після іммобілізації є ключовим етапом *in-situ* біоактивації, що трансформує альгінатні плівки з пробіотиками з пасивних носіїв у функціонально активні біоматеріали. Такий підхід дозволяє досягти високої життєздатності пробіотиків та забезпечує утворення антимікробних сполук безпосередньо в плівці. Запропонована двоетапна стратегія має потенціал для створення високоефективних пробіотичних засобів, зокрема біоактивних ранових покриттів.

Ключові слова: *пробіотики; альгінатний гідрогель; іммобілізація; in-situ культивування; антагоністична активність*

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