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VIABILITY AND IMMOBILIZATION EFFICIENCY OF PROBIOTIC *LACTOBACILLUS BULGARICUS* STRAIN IN ALGINATE FILMS

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Abstract. *The study investigates the production and characterization of alginate films for the immobilization and delivery of the probiotic bacterium Lactobacillus bulgaricus 1Z 03501. Alginate films were synthesized using ionotropic gelation with calcium chloride, and four different protocols were evaluated. The films were characterized in terms of their physical properties, dissolution behavior, and the viability of immobilized bacteria. The results demonstrate that alginate films can be successfully produced using a spraying method, and that the immobilization process does not negatively affect the viability of L. bulgaricus 1Z 03501. The developed alginate films show potential for application as wound dressings for probiotic delivery.*
Key words: alginate films, *Lactobacillus bulgaricus* 1Z 03501, immobilization, probiotics, ionotropic gelation, wound dressing

Probiotic bacteria, when administered in adequate amounts, confer a health benefit to the host, expanding their applications across various fields, including food, pharmaceuticals, and medicine. Notably, the use of probiotics for wound healing has gained attention due to their potential to modulate the wound microenvironment and promote tissue regeneration (Lukic, 2017).

Probiotics, live microorganisms typically found in fermented foods (e.g., yogurt, kefir, sauerkraut) and supplements, offer several health benefits (Gul, 2024). They improve gut health by balancing the microbiota, essential for proper digestion, nutrient absorption, and immune function (Mazziotta, 2023). Probiotics also stimulate the immune system, protect against infections, and reduce inflammation, potentially benefiting conditions like inflammatory bowel disease, arthritis, and asthma (Cristofori, 2021). Emerging research suggests they may also improve mental health by reducing anxiety and depression (Bistas, 2023).

For wound healing, probiotics are particularly beneficial because they create a favorable environment for tissue repair (Bekiaridou, 2021). They produce antimicrobial substances that inhibit harmful bacteria and fungi, reduce inflammation, and promote tissue growth, accelerating healing (Vazquez-Munoz, 2021; Sharma, 2025).

Alginate, a natural polysaccharide derived from brown algae, is widely used for hydrogel and film production due to its biocompatibility, biodegradability, and ease of ionotropic gelation with divalent cations like calcium (Savić Gajić, 2023). Alginate, a complex carbohydrate composed of mannuronic and guluronic acid repeating units, is non-toxic and can be broken down by microorganisms (Murphy, 2023). Alginate films serve as carriers for delivering bioactive agents, including probiotic bacteria, to specific application sites (Medeiros, 2022). These films protect probiotics from harsh gastrointestinal conditions and facilitate controlled release (Vijayaram, 2024).

This study aims to develop and characterize alginate films for the immobilization and delivery of the probiotic bacterium *Lactobacillus bulgaricus* 1Z 03501. Different film preparation

methods were evaluated, and the resulting films were assessed for their physical properties, dissolution behavior, and immobilized bacterial viability.

Materials and Methods

Preparation of sodium alginate solution and alginate films. To prepare a sodium alginate solution, 75 mL of distilled water was combined with 2 g of sodium alginate in a 100 mL glass volumetric flask. The mixture was stirred at room temperature for two hours at 90 rpm using a magnetic stirrer. Once the sodium alginate had swollen, the solution was heated to 50 °C and maintained at this temperature for 30 minutes to facilitate dissolution. After complete dissolution, 6 mL of glycerol was introduced, and the solution was allowed to cool to room temperature. The final volume was then adjusted to 100 mL with distilled water. The prepared solution underwent sterilization by tyndallization, which included three consecutive heating cycles at 100 °C for 60 minutes each, with incubation at 37 °C between cycles.

Alginate films were synthesized using the ionotropic gelation technique, following four previously established methodologies with specific modifications.

The first method involved combining calcium chloride and sodium alginate solutions, as described by Brachkova et al. (2011). A volume of 3 mL of 0.4 M $CaCl_2$ solution, pre-cooled to 4 °C, was introduced into a thermostable silicone mold, followed by the addition of 9 mL of a sodium alginate solution (20 g/L) containing 6% (v/v) glycerol, also pre-cooled to 4 °C. The components were manually stirred using a glass rod, left undisturbed for 15 minutes, and subsequently rinsed with distilled water to eliminate excess calcium chloride ions.

The second method, adapted from Wang et al. (2019), was employed to produce alginate hydrogel films through a rehydration approach. A sodium alginate-glycerol solution was poured into a silicone mold, identical to that used in the first method, to form a layer 4 mm in thickness. This layer was air-dried at room temperature for 24 hours, followed by an additional drying step at 37 °C for one hour. Once dried, the film was rehydrated by adding 10 mL of 0.4 M $CaCl_2$ solution to the mold, allowing ionotropic gelation to occur over a period of 5–10 minutes. The gelled film was then rinsed with distilled water, and any residual moisture on its surface was blotted away using filter paper.

The third method was designed for the fabrication of alginate cryogels. A 4 mm thick layer of sodium alginate solution with glycerol was prepared according to the second method, after which it was cooled to -20 °C and maintained at this temperature for two hours. Following freezing, 10 mL of 0.4 M $CaCl_2$ solution, pre-chilled to 4 °C, was poured over the frozen alginate solution. The sample was left at room temperature for two hours, allowing it to thaw completely and form a cryogel. The resulting cryogel film was then transferred to a separate container, where an additional 10 mL of 0.4 M $CaCl_2$ solution was added. After a 15-minute incubation, the film was thoroughly washed with distilled water.

The fourth method involved aerosol treatment of the sodium alginate solution with calcium chloride. For this purpose, a sodium alginate solution (10 or 20 g/L) with or without glycerol (6%, v/v) was dispensed into silicone molds with a square bottom (42 × 42 mm) in volumes of 1.74, 3.33, 5.29, and 7.1 mL, forming layers of 1-, 2-, 3-, and 4-mm thickness, respectively. The molds were leveled horizontally, and a 0.4 M calcium chloride solution was sprayed onto the surface. The samples were left to react for 5–20 minutes and washed with distilled water.

Evaluation of film dissolution in chelating agent solutions. Each obtained film was cut in half with scissors, yielding rectangular fragments approximately 20 × 40 mm in size. The fragments were then immersed in penicillin vials containing 20 mL of distilled water and left for 10 minutes to remove residual calcium chloride solution.

The washed film fragments were transferred to clean penicillin vials, where 10 mL of either ethylenediaminetetraacetic acid (EDTA, 40 g/L) or sodium citrate (SC, 40 g/L) solution was added. The samples were maintained at 20 °C with periodic shaking. The dissolution time of the film samples was recorded, with dissolution assessed visually.

Immobilization of *L. bulga*ricus culture in alginate films. A sterile sodium alginate solution (30 g/L) was used as a stock solution. To prepare alginate hydrogel films containing immobilized probiotic bacteria, 2 mL of the sodium alginate stock solution was drawn into a sterile disposable syringe and transferred to a sterile vial. Then, 4 mL of a *L. bulgaricus* 1Z 03501 starter liquid culture in Blaurock medium was added and mixed. A series of 100-fold serial dilutions of both the starter culture and the alginate-bacteria mixture were prepared in Blaurock medium, incubated for 48 hours at 37 °C, and analyzed to determine the concentration of viable cells (CFU/mL).

The alginate-bacteria mixture was then dispensed into sterile silicone molds with a square bottom (42 mm per side) in 3.33 mL portions, forming a layer approximately 2 mm thick. The molds were leveled horizontally. A sterile 0.4 M calcium chloride solution was sprayed onto the surface and left for 15 minutes to facilitate gelation. The resulting films were visually inspected and photographed.

Each film was transferred to a separate 100 mL vial containing 20 mL of sterile distilled water and incubated for 30 minutes with continuous stirring at 60 rpm on a shaker to remove residual calcium chloride. Then each film was transferred to a 50 mL vial containing 20 mL of sterile sodium citrate solution (40 g/L) and kept at room temperature (20 °C) with periodic shaking.

Samples were collected from the water used to wash the film and from the solution obtained after dissolving the film in sodium citrate. A series of serial dilutions were prepared to determine the concentration of viable cells. The cultures were incubated in Blaurock medium in test tubes at 37 °C for 48 hours, after which colony counts were performed.

Statistical analysis. Data are presented as the mean ± standard deviation (SD) unless otherwise specified. Statistical analysis was performed using the IBM SPSS statistical software. The significance of differences between groups was determined by one-way ANOVA followed by Tukey's post hoc test. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. Statistically significant differences ($p<0.05$) are indicated with an asterisk (*) All experiments were conducted in triplicate, and data were analyzed from three independent experiments.

Results. Through the addition of sodium alginate solution to a calcium chloride solution according to the first protocol, an amorphous structure of approximately 4 cm in diameter with a gel-like consistency of about 5 mm thickness was obtained, exhibiting a round shape but lacking the appearance of a film. This structure maintained its shape and linear dimensions for 10 days when kept in sterile distilled water at 8 °C.

Under the second protocol, namely by performing ionotropic gelation during the rehydration of the dry sodium alginate film in a calcium chloride solution, elastic and strong alginate film with a thickness of approximately 1 mm was obtained. This film retained its mechanical properties for 10 days when immersed in sterile distilled water at a temperature of 8 °C.

Following the protocol for preparing alginate cryogel, soft-to-touch, sponge-like porous alginate film with a thickness of approximately 2-3 mm was obtained, which maintained their mechanical properties for 10 days when kept in sterile distilled water at a temperature of 8 °C albeit with a reduction in linear dimensions observed during this period.

Using the fourth method, films with varying qualities were obtained, dependent on the concentration of sodium alginate and the thickness of the solution layer.

Treatment of a 20 g/L sodium alginate solution resulted in films with unsatisfactory mechanical properties, regardless of layer thickness (1 to 4 mm). Specifically, the film obtained from a 1 mm thick solution layer exhibited uneven thickness (Fig. 1, a). In layers 2 to 4 mm thick, unpolymerized solution was observed within the films (Fig. 1, b-d). It is hypothesized that these unsatisfactory results were due to both the high concentration of sodium alginate and

the presence of glycerol, which may interfere with the formation of ionic crosslinks between calcium ions and the carboxyl groups of alginic acid.

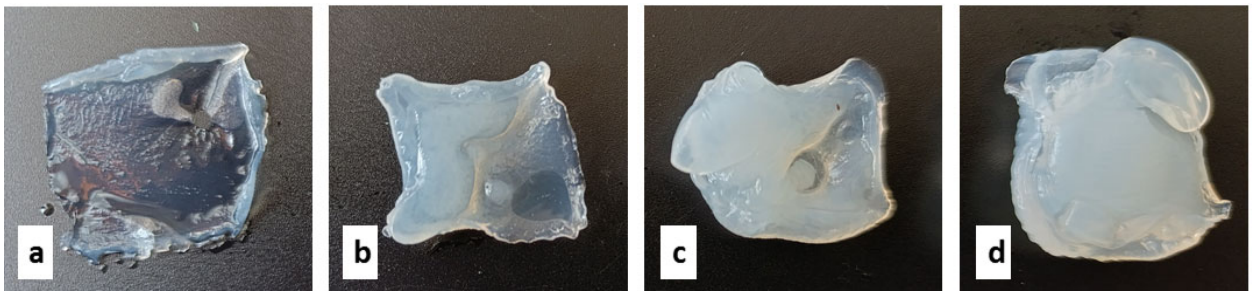

Fig. 1. Alginate film samples obtained by spraying calcium chloride solution onto a sodium alginate solution with a concentration of 20 g/L and thicknesses of 1 (a), 2 (b), 3 (c), and 4 (d) mm

Aerosol treatment of sodium alginate solutions (both 10 and 20 g/L) with calcium chloride, using a 1 mm thick solution layer, resulted in transparent films with uneven thickness (Fig. 2, a, c). However, when using a 2 mm thick solution layer and a 10 g/L sodium alginate concentration, a film of uniform thickness and regular shape was produced (Fig. 2, b). Conversely, at a 20 g/L sodium alginate concentration with a 2 mm layer, unpolymerized liquid sodium alginate solution remained within the film after treatment, leading to film deformation (Fig. 2, d). Therefore, based on visual characteristics, the film formed from a 10 g/L sodium alginate solution with a 2 mm layer thickness was selected for further studies.

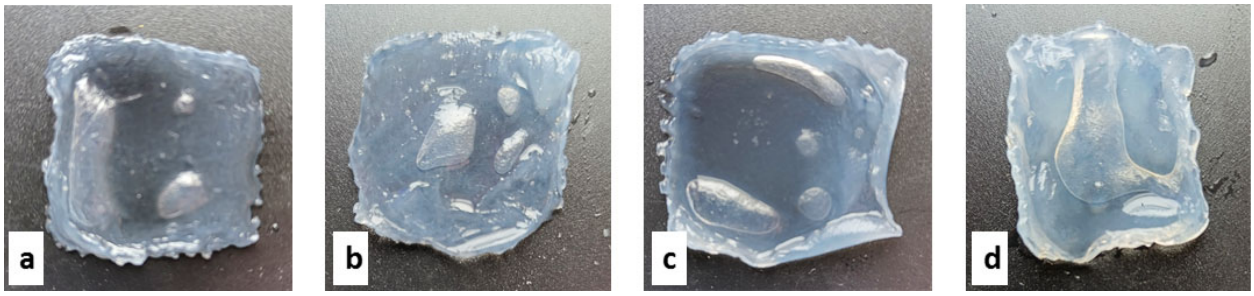

Fig. 2. Alginate film samples obtained by spraying calcium chloride solution onto a sodium alginate solution with a concentration of 10 (a, b) or 20 (c, d) g/L and thicknesses of 1 (a, c) or 2 (b, d) mm

Both sodium citrate and EDTA effectively dissolved alginate films, with the dissolution rate inversely proportional to the initial sodium alginate concentration and layer thickness. Notably, the concentration of the sodium alginate solution had a more significant impact on the dissolution rate than its thickness. Furthermore, EDTA exhibited a higher dissolution rate than sodium citrate. Complete dissolution was observed within 12–38 minutes (fig. 3).

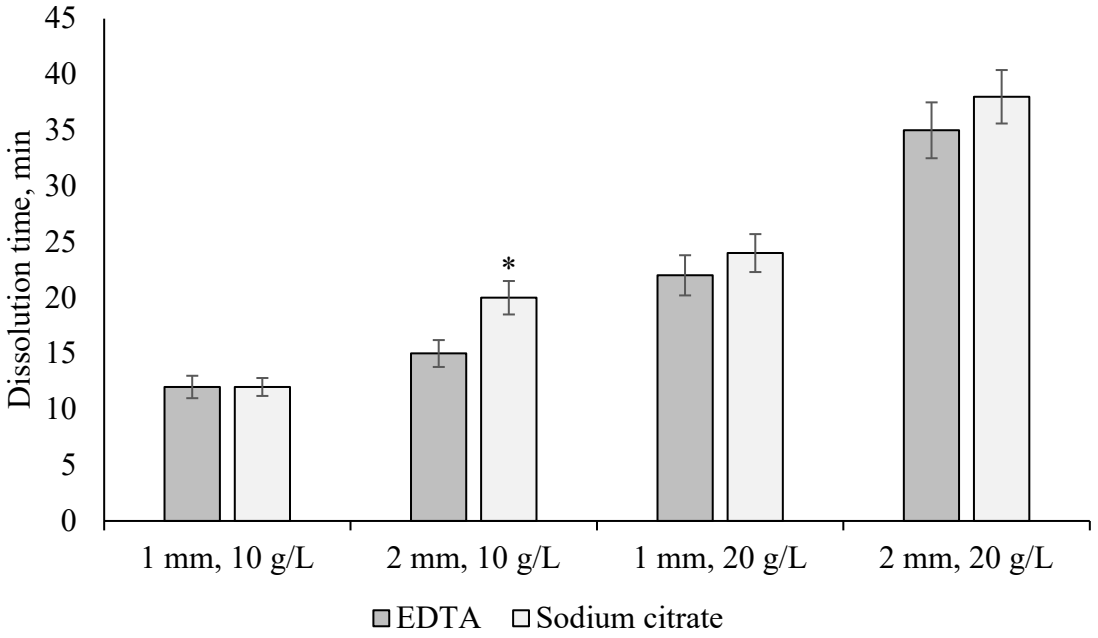

Fig. 3. Time required for alginate films dissolution (min) depending on sodium alginate solution concentration, layer thickness, and chelating agent composition.

The starter culture contained (1,2±0,1)×10^9 viable *L. bulgaricus* 1Z 03501 cells per mL (fig. 4, a). Mixing the culture with the sodium alginate solution did not result in a decrease in the CFU concentration, calculated per fraction of the culture incorporated into the film, which was (1,6±0,1)×10^9 CFU/mL (fig. 4, b).

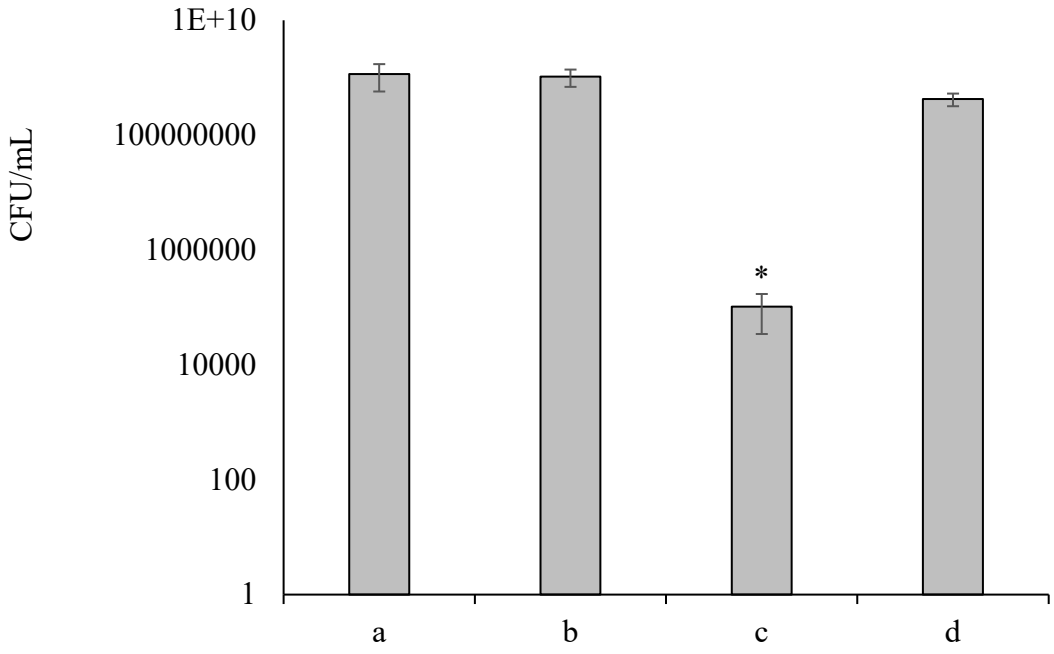

Fig. 4. Viable cell counts of *L. bulgaricus* 1Z 03501 (CFU/mL): (a) initial culture, (b) culture/sodium alginate mixture, (c) film wash (distilled water), (d) film dissolution (sodium citrate).

After complete dissolution of the calcium alginate film in sodium citrate solution, the concentration of viable *L. bulgaricus* 1Z 03501 cells was (1.4±0,2)×10^9 CFU/mL, or 4.2×10^8

CFU per 1 mL of film volume. This represents about 40% of the cell concentration in the sodium alginate mixture with the starter culture prior to gelation. It is hypothesized that the loss of cells occurred during the calcium chloride solution fixation of the sodium alginate gel. In our opinion, this cell loss is insignificant. Nonetheless, the film production method developed in this study, which involves spraying a fixing solution onto the surface of the sodium alginate mixture, requires further optimization to minimize probiotic cell loss.

The wash water from the calcium alginate-based films contained $(1.7\pm0.1)\times10^4$ CFU/mL. This corresponds to a total of $(3.4 \pm 0.2)\times10^5$ CFU washed from a single film, which is equivalent to $(1.0\pm0.1)\times10^5$ CFU per 1 mL of film. This represents approximately 0.02% of the cell concentration within the film (4.2×10^8 CFU/mL).

Discussion. The first protocol, involving the addition of sodium alginate solution to calcium chloride solution, resulted in an amorphous structure rather than a defined film, rendering it unsuitable for wound dressings. This indicates that simple mixing does not provide sufficient control over the gelation process for uniform film formation. This finding aligns with the understanding that controlled ionotropic gelation is essential for stable and mechanically robust alginate structures. Factors such as ion diffusion rate and mixing method significantly influence gelation kinetics and hydrogel properties.

Hydrogels produced using the second (rehydration) and third (cryogel) protocols met the mechanical and organoleptic requirements for wound dressings. The rehydration method yielded a tough film, while the cryogel method resulted in a porous, sponge-like structure. This difference is attributed to distinct gelation mechanisms and structural changes induced by each method. The rehydration method likely creates a denser polymer network, contributing to toughness. Conversely, the cryogel method's freezing process forms ice crystals that, upon thawing, leave pores within the alginate matrix (Tan, 2021).

Given its relatively high toughness, the rehydration method appears suitable for producing alginate wound dressings with immobilized therapeutic agents, such as probiotic microorganisms, bacteriophages, or nanoparticles, for infected wound treatment. The film's toughness would provide a protective barrier, while the immobilized agents offer targeted therapy.

The cryogel method is convenient for producing porous, sponge-like hydrogels that can be seeded with cell preparations (fibroblasts, mesenchymal stem cells) to impart regenerative properties to wound dressings (Razavi, 2019). The porous structure facilitates cell infiltration and proliferation, making cryogels attractive for tissue engineering applications (Carriero, 2023).

The fourth protocol, spraying calcium chloride onto the alginate solution, proved most suitable for immobilizing probiotic bacteria. It avoids steps that could compromise bacterial viability and enables rapid production of alginate films with satisfactory mechanical characteristics. The aerosol application of calcium chloride likely promotes uniform and gentle gelation, minimizing damage to encapsulated bacteria.

Thus, spraying a fixing solution onto a sodium alginate/*L. bulgaricus* 1Z 03501 culture produced an alginate film with mechanical properties suitable for gel wound dressings. The ionotropic gelation method did not negatively affect bacterial cell viability, a crucial finding for therapeutic efficacy. Furthermore, this method demonstrated high efficiency in incorporating the probiotic culture, good bacterial cell retention, and low cell release during washing. The high encapsulation efficiency indicates effective bacterial entrapment within the alginate matrix. The low cell release confirms good retention, essential for controlled delivery.

Conclusions. Immobilization of viable *L. bulgaricus* 1Z 03501 in alginate films, by mixing a cell suspension with 10 g/L sodium alginate solution followed by calcium chloride aerosol treatment, demonstrates high cell inclusion during ionotropic gelation and low cell loss during leaching. This method offers a promising approach for developing alginate-based wound dressings for probiotic delivery. Efficient immobilization and retention of viable probiotic cells

within alginate films opens possibilities for novel therapeutic applications in wound healing and other medical areas. Future research should focus on optimizing film properties, evaluating *in vivo* efficacy, and exploring combination therapies with other wound healing agents.

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ЖИТТЄЗДАТНІСТЬ ТА ЕФЕКТИВНІСТЬ ІММОБІЛІЗАЦІЇ ПРОБІОТИЧНОГО ШТАМУ *LACTOBACILLUS BULGARICUS* В АЛЬГІНАТНИХ ПЛІВКАХ

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***Резюме**. Розроблено метод отримання альгінатні плівки для іммобілізації пробіотичного штаму Lactobacillus bulgaricus 1Z 03501. Плівки були отримані методом іонотропного гелеутворення з використанням хлориду кальцію за чотирма оптимізованими протоколами. Досліджено фізичні властивості плівок, кінетику їх розчинення та вплив на життєздатність іммобілізованих бактерій. Встановлено, що аерозольне нанесення розчину хлориду кальцію на розчин альгінату натрію є оптимальним методом формування плівок. Показано, що іммобілізація не знижує життєздатність L. bulgaricus 1Z 03501. Альгінатні плівки з пробіотиками, отримані в даній роботі, є перспективними для використання в якості ранозагоювальних покриттів.*

Ключові слова: альгінатні плівки, *Lactobacillus bulgaricus* 1Z 03501, іммобілізація, пробіотики, іонотропне гелеутворення, ранозагоювальне покриття.

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