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CREATION OF AN IMMOBILIZED PROBIOTIC BASED ON BACTERIAL CELLULOSE FOR THE CORRECTION OF THE INTESTINAL MICROBIOME

The structural features of bacterial cellulose (BC) make it very popular to use it for creating composite materials. Immobilization of bacteria of the *Bacillus* genus in BC polymer gives the carrier matrix new functional properties that help restore and maintain a favorable balance of microflora in the gastrointestinal tract. The addition of sodium alginate had a positive effect on the synthesis of BC by *K. xylinus* C-3, providing a polymer yield of 12.11 g/l. The "adsorption-incubation" method was used for surface and spatial immobilization of *Bacillus subtilis*, which provides a high concentration of cells – 10^8 CFU per 1 g of carrier. The BC/*Bacillus* biocomposite inhibited the growth of *E. coli, S. typhi, S. typhimurium, Citr. aerogenes, and P. vulgaris* up to 85.4% after 24 hours of contact, and after 72 hours completely inhibited the growth and viability of test organisms in *vitro*. Immobilized probiotics significantly exceeded the suspension of free microbial ells in terms of resistance to gastric juice. The activity of probiotics *in vivo* determined on models of experimental dysbiosis. Probiotic microgranules of BC/*B.subtilis* can be used to correct the microbiocenosis of the large intestine.

Key words: bacterial cellulose, *B. subtilis*, dysbiosis.

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Ішек микробиомасын түзету үшін бактериялық целлюлоза негізінде иммобилизацияланған пробиотик жасау

Құрылымдық қасиеттерінің ерекше болғандығына байланысты композициялық материалдарды жасау үшін бактериялық целлюлозаны (БЦ) қолданған ыңғайлы. *Bacillus* тектес бактериялардың БЦ полимеріне иммобилизациялануы, тасымалдағыш матрицаға асқазан-ішек жолындағы микрофлораның тепе-теңдігін қалпына келтіруіне және сақтауға ықпал ететін жаңа функционалдық қасиеттер береді. БЦ-ға натрий альгинатының қосылуы *K. xylinus* С-3 синтезіне оң әсерін берді және 12,11 г/л полимер шығымын қамтамасыз етті. «Адсорбция-инкубация» әдісі *Bacillus subtilis*-тің беткі және кеңістіктік иммобилизациясы үшін қолданылды, бұл 1 г тасымалдаушыға 10^8 КТБ/ 1 г тасымалдағышқа жоғары жасуша концентрациясын қамтамасыз етеді. BC/*Bacillus* биокомпозиті 24 сағаттық байланыста *E. coli, S. typhi, S. typhimurium, Citr. aerogenes, P. vulgaris* өсуін 85,4%-ға дейін басады, ал 72 сағаттан кейін *in vitro* жағдайында тест-бактериялардың өсуі мен өміршеңдігін толығымен тежеді. Иммобилизацияланған пробиотиктер асқазан сөліне төзімділігі бойынша бос микроб жасушаларының суспензиясына қарағанда айтарлықтай жоғары болды. Пробиотиктердің белсенділігі *in vivo* эксперименттік дисбиоз үлгілері арқылы анықталды. Тоқ ішектің микробиоценозын түзету үшін БЦ/*B.subtilis* пробиотикалық микрогранулаларын қолдануға болады.

Түйін сөздер: бактериалды целлюлоза, *B. subtilis*, дисбактериоз.

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Создание иммобилизованного пробиотика на основе бактериальной целлюлозы для коррекции кишечного микробиома

Структурные особенности бактериальной целлюлозы (БЦ) обеспечивают большую популярность ее использования для создания композитных материалов. Иммобилизация бактерий рода *Bacillus* в полимере БЦ придает матрице-носителю новые функциональные свойства, способствующие восстановлению и поддержке благоприятного баланса микрофлоры в ЖКТ. Внесение альгината натрия положительно влияло на синтез БЦ *K. xylinus* С-3, обеспечивая выход полимера – 12,11 г/л. Использован метод «адсорбции-инкубации» для поверхностной и пространственной иммобилизации *Bacillus subtilis*, обеспечивающий высокую концентрацию клеток – 10^8 КОЕ на 1 г носителя. Биокомпозит БЦ/*Bacillus* подавлял рост *E. coli, S. typhi, S. typhimurium, Citr. aerogenes, P. vulgaris* до 85,4% после 24-часового контакта, а по истечении 72 часов полностью ингибировал рост и жизнеспособность тест-организмов в условиях *in vitro*. Иммобилизованные пробиотики по уровню резистентности к воздействию желудочного сока значительно превосходили суспензию свободных клеток микроорганизмов. Активность пробиотиков в условиях *in vivo* определяли на моделях экспериментального дисбактериоза. Пробиотические микрогранулы БЦ/*B. subtilis* могут быть использованы для коррекции микробиоценоза толстого кишечника.

Ключевые слова: бактериальная целлюлоза, *B. subtilis*, дисбактериоз.

Abbreviations

BC – bacterial cellulose, gastrointestinal tract – gastrointestinal tract, GPB – granules with probiotics

Introduction

The design features of bacterial cellulose (BC): micro- and nanofibrillar structure, high porosity and crystallinity create a huge potential for creating various composite materials based on it [1-2]. A characteristic structural feature of the BC is that the aggregates of fibrils occupy an insignificant part of the volume, which makes it possible to introduce into the BC not only a variety of substances, including those with biological activity, but also cells of prokaryotic or eukaryotic organisms. They give the cellulose carrier matrix new functional properties.

One of the promising areas of modern biotechnology is the development of medicines based on biologically active substances produced by bacteria, including representatives of the *Bacillus* genus [3]. According to recent studies, the use *of Bacillus* species as probiotics has gained great interest [4]. These bacteria are effective in preventing respiratory infections and gastrointestinal disorders, as well as in overcoming symptoms associated with irritable bowel syndrome [5-6]. The presence *of B. subtilis* contributes to the maintenance of a favorable balanced microbiota in the gut and enhances the growth and viability of probiotic cells of lactic acid bacteria [7]. It has also been suggested that these probiotic properties are related to its ability to stimulate the immune system [5] and the production of antimicrobial substances [8-9], or even to induce signal interference against pathogens [10]. Such purposeful correction of disturbed biochemical and physiological processes by introducing the necessary saprophytic bacteria that produce biologically active substances is commonly called the “microbial saprophytic pharmacopoeia”.

Successful immobilization of bacteria [11-13] on BC with the preservation and even increase of their physiological activity served as the basis for conducting this study aimed at creating a biocomposite material with antimicrobial and probiotic properties by including bacterial cells of the *Bacillus* genus in BC. **The aim of the study is** to develop an immobilized probiotic for the correction of microecological disorders in the intestine.

Materials and Methods

2.1 Research objects

1. *Komagataeibacter xylinus* C-3 – BC strain producer;

2. *Bacillus subtilis* 3H strain isolated from Bactisporin preparation;

3. Test microorganisms, obtained from the American type culture collection: *E.coli, S.typhi, S.typhimurium, Citr.aerogenes, P.vulgaris*.

4. 40 male mongrel rats weighing 180-220 g.

2.2 Obtaining BC in submerged culture cultivation conditions

Cellulose synthesis by strains of acetic acid bacteria was carried out on nutrient media containing aqueous solutions of yeast extract, glucose, peptone, ethanol, and NaAlg at concentrations determined as a result of optimizing the nutrient medium with a pH of 5.9-6.0. The inoculum was a 48-hour culture of acetic acid bacteria grown on a medium containing yeast extract and beer wort in a ratio of 1: 1 with 2 wt. % glucose 1 vol. % of ethanol. Cultivation was performed on a Biosan ES-20 orbital thermoshaker at 30°C for 7 days. The globules were then washed from the NaOH solution with distilled water, 0.5% acetic acid solution, and again distilled water until a neutral reaction was achieved. The resulting globules were stored in distilled water at 5°C. The biomass of BC globules was determined after preliminary drying in a dry-fire thermostat at 80°C to a constant sample weight.

2.3 Immobilization of Bacillus subtilis 3H bacteria into BC matrix by "adsorption-incubation" method

At the stage of "adsorption", the biomass of a 48-hour culture of *Bacillus subtilis* bacteria was suspended in a phosphate buffer solution (cell titer 10^{-10} CFU/ml). BC granules were autoclaved at 0.35 bar at 110° C for 30 minutes. Then BC granules were added to the cell suspension and incubated for 96 hours with continuous weak stirring (120 rpm). The liquid was decanted, and the globules were washed from weakly attached cells with sterile saline solution.

At the "incubation" stage, BC with immobilized bacterial cells were placed in sterile Erlenmeyer flasks with liquid nutrient broth and incubated at 30 °C for 96 hours.

After the immobilization procedure, the wet granules were freeze-dried at 40 °C, 5 mbar for 48 hours (Telstar LyoQuest-55, Spain). After lyophilization, 10 ml of saline solution was added to each sample and cell viability was determined by seeding serial dilutions of the resulting suspension on nutrient agar. Colony forming units were counted after 24-hour incubation at 37 °C.

2.4 Determination of the number of live cells in BC granules

Granules with bacterial cells were washed with sterile saline solution and cleaved with the cellulase enzyme (100 ml/1 0.05 M citrate buffer, pH 4.8, Sigma Aldrich). The bacterial suspension obtained by splitting the globules with cellulase was suspended in 1 ml of a phosphate buffer solution. The number of immobilized microorganisms was determined by two methods: (a) by the optical density of the culture suspension at a wavelength of 650 nm (Infinite 200 PRO NanoQuant, Tecan, Switzerland) and (b) by inoculation of consecutive ten-fold dilutions on nutrient agar (Biocorp). After incubation of the dilutions for 24 h at 37 ° C, the grown colonies were counted and the content of live bacteria in 1 g of cellulose was calculated. The optical density of the bacterial suspension was used to determine the number of viable and dead bacteria, while inoculation on bacteriological media allowed us to determine only viable microbial cells.

The efficiency of immobilization of bacterial cells in BC was quantified by the method described by Leboffe and Pierce [14]. The average cell density (cells/g) was calculated as follows: the number of cells immobilized inside the BC polymer + the number of cells adsorbed on the BC surface/ weight of the cellulose film. Immobilization efficiency, % = total number of cells immobilized on BC/total number of cells in the inoculum * 100%.

2.5 Determination of the antagonistic activity of BC granules

The antagonistic activity of BC granules based on it was determined by the method of co-cultivation of target microorganisms with experimental samples (Time-kill test).

This test is described in detail in the M26-A CLSI document [15]. BC biocomposites weighing 1 gram were placed in test tubes with a liquid nutrient medium inoculated with target microorganisms with a cell concentration of 5 x 10^5 CFU / ml, and then incubated in a shaker at 37°C for 24 hours. The medium with the test strain without adding BC samples was used as a control. The percentage of dead cells relative to the growth control was then calculated at various time intervals (0, 1, 6, 10, and 24 hours). To do this, 50 µl of bacterial suspension was taken from each tube, inoculated on nutrient agar, and incubated at 37 °C for 48 hours to count colony-forming units and determine the number of living cells (CFU/ml). The decrease in the number of bacteria was estimated by the following formula: R (%): AB/A x 100%, where R is the percentage of the number of bacteria, A is the number of bacteria in the control group (without adding cellulosic materials), and B is the number of bacteria grown in test tubes with biocomposites. The bactericidal effect of samples is considered when 90% of cell lethality is achieved in 6 hours, which is equivalent to 99.9% of bacterial lethality in 24 hours [16].

2.6 Induction of experimental dysbiosis in rats

Before the introduction of the antibiotic, feces of intact animals were plated on differential diagnostic

media to determine the content of the main groups of indigenous bacteria in the intestines of rats.

Starting from the next day, after the preliminary study, animals divided into 4 groups (5 individuals each) received ampiox intragastrically for five days at a dose of 40 mg per day for 5 days. The first group received only an antibiotic, the second – BC/*Bacillus* microgranules after etiotropic therapy, the third – free *Bacillus cells* after taking ampiox, the fourth group – intact animals.

The analysis was performed 5 days later (immediately after discontinuation of the antibiotic) and 20 days after the start of the experiment. The main criteria for the bacteriological effectiveness of this method were the population level of bifidobacteria, lactobacilli and *Escherichia* with unchanged enzymatic properties, as well as qualitative and quantitative characteristics of the content of opportunistic microorganisms in feces. The results of microbiocenosis correction were evaluated by the traditional bacteriological method [17].

Results and Discussion

3.1 Preparation of bacterial cellulose globules with probiotic bacteria

The most general definition of probiotics is microorganisms that, when administered in adequate amounts, have a beneficial effect on the host's body. Their beneficial effect is achieved by restoring and maintaining the gut's own microflora, i.e. they eliminate dysbiotic disorders and associated gastrointestinal dysfunctions [18]. Recently, bacteria of the *Bacillus* genus, especially *B. subtilis*, have gained great interest as probiotic microorganisms due to their ability to maintain a favorable balance of microflora in the gastrointestinal tract. This is due to the antagonism of *B. subtilis* against a wide range of pathogenic and opportunistic microorganisms, self-elimination from the gastrointestinal tract, stimulating effect on digestion, anti-allergenic, antitoxic, sanitizing and restorative effects on the body [19; 4]. In this regard, the second way of using BC considered in this paper is the development of an immobilized probiotic for the correction of microecological disorders in the intestine.

Probiotics are available in various forms: food, capsules, sachets, or tablets, but what they have in common is that they are administered orally [20-21]. To be able to influence the gut microbiota, probiotic microorganisms must survive in the consumed food during the shelf life and transit period in acidic conditions of the stomach and resist degradation by hydrolytic enzymes and bile salts in the upper intestine. Available information on the viability of probiotics containing *Bacillus* bacteria in the gastrointestinal tract is contradictory. On the one hand, since *B. subtilis* spores are able to survive in conditions of extreme pH and low oxygen content, a large number of dormant but viable microbes can reach the lower parts of the intestine and carry out beneficial effects [7]. On the other hand, a number of studies have established that not all the introduced bacteria reach the target niche – the large intestine [8]. Providing probiotic living cells with a physical barrier against adverse conditions is an approach that is currently receiving considerable interest. One of these methods is their immobilization on a polysaccharide matrix. A promising matrix is bacterial cellulose (BC), which is attracting increasing interest from the scientific community due to its suitability for cell immobilization processes, including probiotics. Researchers working on this problem immobilized strains of *Lactobacillus spp*. It was found that this method provided high-level protection of these microorganisms from the effects of gastric juice and bile salts [22-23]. However, a view of the available literature has shown that so far no studies have been conducted to assess the feasibility of using BC as a carrier for immobilizing *Bacillus* bacteria.

The use of probiotics implies their use *per os*. In this regard BC granules in the form of granules were obtaied, in which probiotic bacteria were included by spatial immobilization.

Cultivation conditions affect the morphology of BC. Under static conditions, bacteria accumulate on the oxygen-rich surface of the nutrient broth to form intertwined ribbons with a low organizational structure. This morphology can be improved by producing BC in a submerged agitated culture, where the bacteria are well dispersed in the suspension of the culture medium. Under such cultivation conditions, cellulose can be obtained as a suspension of fibrils, irregular globules, granules or spheres.

BC production by this method was carried out under mixing conditions on an orbital thermoshaker Biosan ES-20 at 180 rpm.

It is believed that the submerged method of obtaining BC allows achieving higher productivity [24-25]. However, BC is an insoluble polymer in water, so with increasing its concentration, the viscosity of the culture medium increases.

Therefore, when cultivating producers in this way, the cellulose synthesized by them often forms large lumps, irregular globules, and amorphous filaments. This leads to a decrease in the supply of

nutrients and oxygen to bacterial cells. As a result, the yield of cellulose decreases, and it can often have an uneven structure and altered properties.

Recently, there have been several reports that the addition of water-soluble polymers such as xanthan, agar, polyacrylamide-co-acylic acid, and acetate can reduce the shear stress, i.e., prevent BC coagulation during cultivation, inducing the formation of homogeneous small granules [26-27].

Given that sodium alginate is also a water-soluble polysaccharide, and since it contains many -COOH and -OH groups, it was suggested that adding it to the culture medium can stimulate the level of synthesis and obtain a polymer of regular structure. To determine the effect of sodium alginate, the BC producing strain was cultured in a medium with different concentrations of NaAlg and without it.

The results of the experiment on obtaining BC by strain of *K.xylinus* C-3 in a medium containing NaAlg in the concentration range of 0-0.1% are shown in Figure 1.

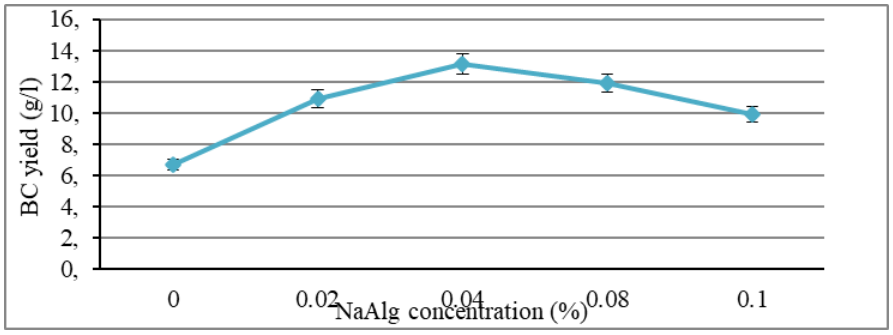


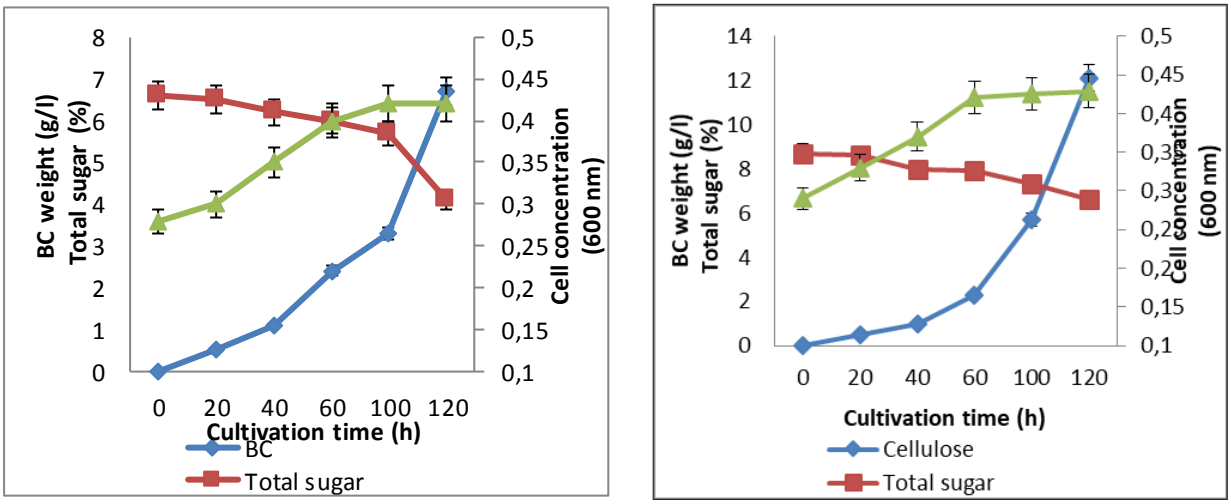
Figure 1 – Effect of adding sodium alginate to the medium on the synthesis of bacterial cellulose under submerged conditions

It was found that the introduction of alginate has a positive effect on the formation of BC by *K. xylinus* C-3. Moreover, the optimal NaAlg concentration is 0.04%. So, if the BC yield in the medium without sodium alginate was 6.7 g/l, then in the presence of 0.04% NaAlg in the medium, the synthesis was significantly higher – 12.11 g/l. It should be noted that a further increase in the concentration of sodium alginate to 0.1% leads to a gradual decrease in the level of BC synthesis. Most likely, a higher concentration of sodium alginate increases the viscosity of the medium, which reduces the efficiency of biosynthesis of this polymer by the producing strain.

The time intervals of cellulose production, cell concentration, and total sugar content in the mixed cultures are shown in Figure 2.

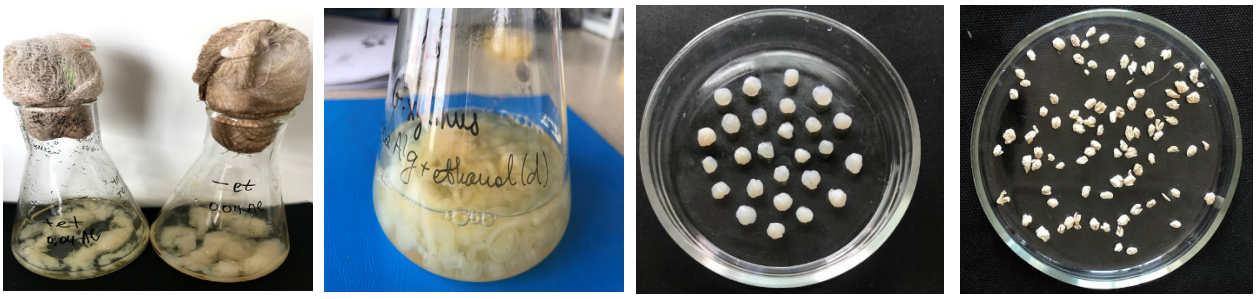
In the absence of NaAlg, cells grew exponentially after a 24-hour lag period and linearly between 48 and 72 hours of culture time, then reached a stationary phase. However, the addition of 0.04% NaAlg shortens the lag period and accelerates cell growth in the early phase of culture. In addition, when the culture reached the logarithmic period, cellulose also began to form and increase due to continuous cell growth regardless of the presence of NaAlg in the medium. Total sugar consumption in the medium with and without sodium alginate was similar. However, the total sugar consumption *of K. xylinus* C-3 in the medium with the addition of 0.04% NaAlg was 2.0%, than 1.5% in the control medium without NaAlg. In addition, the final cellulose yield was 27% of the total sugar initially added in the presence of 0.04% NaAlg, compared to 24% in the control. Thus, the addition of 0.04% NaAlg promoted cell growth and enhanced BC production in shaker flasks.

BC formed in submerged conditions in a medium without alginate is a large oval or irregular globules, may look like disordered and fibrous clusters, large lumps (Figure 3A). When alginate is added, small oval globules are formed. Cellulose globules formed in a stirred culture in a medium with the addition of NaAlg are shown in **Figure 3 B.** The globules were separated and periodically washed with 0.5-1% aqueous NaOH solution at boiling until the cells were removed. The cellulose globules were then washed from the NaOH solution with distilled water, 0.5% acetic acid solution, and again distilled water until a neutral reaction was achieved. The resulting cellulose globules were stored in distilled water at 5°C.



A B

Figure 2 – Dynamics of cellulose synthesis by *Komagataeibacter xylinus* C-3 strain in submerged culture (A – standard medium; B – jmedium with NaAlg)



A B C D

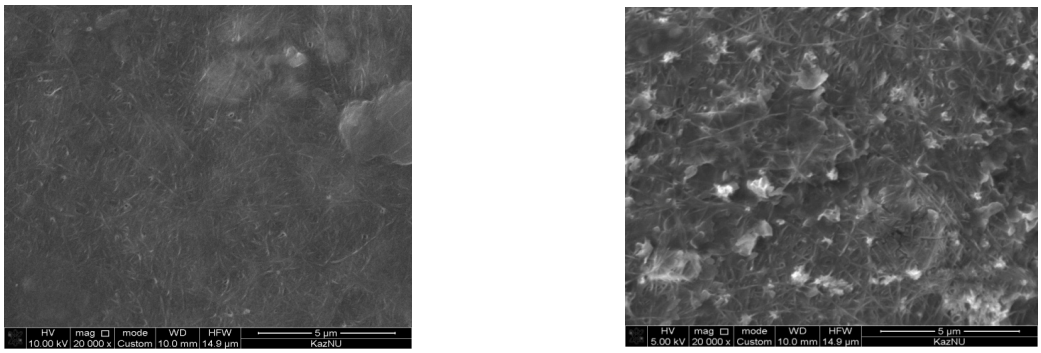
Figure 3 – BC globules formed under submerged cultivation conditions (A – BC globules obtained during cultivation of the producer without alginate; B – with the addition of alginate; C – wet globules after purification; D – dried cellulose samples)

Alginate can reduce the viscosity of the broth, reducing the shear stress, and, due to this, prevent BC coagulation during cultivation. Small granules formed in the alginate medium are more advantageous for transferring nutrients and oxygen to bacterial cells located inside and on the surface of the cellulose matrix. This is one of the reasons for stimulating BC synthesis in a medium with alginate or other water-soluble polysaccharides [26].

Cellulose synthesized during agitated culture can often have an uneven structure and altered properties: a lower degree of polymerization, mechanical strength, and crystallinity than those produced during static cultivation [27-28]. Since the structure of cellulose varies depending on the state of the culture, such as additives, the structural characteristics of BC obtained in media with and without NaAlg are compared using scanning electron microscopy (SEM). The structural characteristics of the BC synthesized in the medium with and without NaAlg addition were analyzed using a scanning electron microscope are shown in **Figure 4.**

The fibers of the film obtained under agitated culture conditions in the control medium without NaAlg were characterized by a more compact and strongly elongated structure than in the medium with the addition of NaAlg.

There were a lot of NaAlg particles on the surface of the matrix. The inner layer consisted of many ultrathin fibrils that were bent and twisted with each other. The fibers had a large width. Since the width of the fibers affects such properties of BC as water retention and mechanical strength, the consumer qualities of this type of cellulose increase. This will expand the scope of application of the BC.



A B

Figure 4 – Scanning electron micrographs of bacterial cellulose obtained under agitated culture conditions (A – standard medium; B – medium with NaAlg)

BC obtained in a medium with the addition of sodium alginate consisted of a three-dimensional matrix with a large number of pores. Compared to smooth surfaces, the three-dimensional structure of BC creates favorable conditions for cell adhesion. The number of pores in the BC is influenced by the conditions of post-processing, which is carried out to purify it. A recent study showed an increase in total porosity after alkaline treatment of the BC with NaOH. This was due to the fact that this purification helps to release the space normally occupied by producer cells [29]. The addition of porogens to the growing culture during the BC biosynthesis process can regulate the pore size and porosity of the final structure. An increase in the pore diameter was observed when alginate was incorporated into the BC structure [30]. This may be due to the breakdown of hydrogen bonds between cellulose fibers due to mixing with another component.

To immobilize the *Bacillus* cells, the "adsorption-incubation" method described was used. In wet and dry globules, the BC weight was 1 mg and 15 mg, respectively. The size of the granules is 130-140 microns, i.e., in fact, they are microcapsules of the matrix type. The difference between the weight of the wet and dry forms of BC was mainly due to water absorption. It was found that a greater number of immobilized microorganisms were registered in the wet BC (Figure 5).

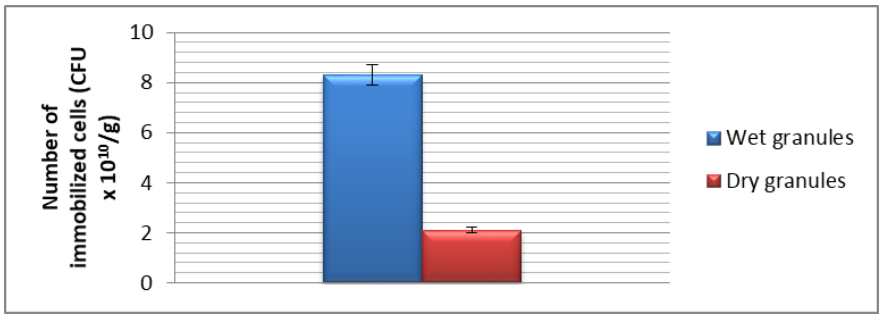


Figure 5 – Efficiency of immobilization of *Bacillus subtilis 3H* cells in BC globules

This phenomenon can be explained by differences in the location of fibrils in wet and dry BC. From the chemical point of view, BC is highly hydrophilic and therefore can absorb a large number of water molecules, whereas from the physical point of view, BC is a three – dimensional network with a large number of pores [31]. In the hydrated form, BC exhibits higher water absorption, lower density, and less interconnected microfibrils compared to its dry form, which can promote cell penetration into deeper layers of the carrier [32]. Differences in the number of immobilized cells may also result from the developed

porosity of wet BC cells. The porous structure of wet cellulose provides more space for immobilization and facilitates the diffusion of substrates from the medium into the interior of the carrier structure, providing a moist, favorable environment for cells [33-34]. It is also well known that BC synthesized under various cultivation conditions (static or mixed) is characterized by various physical properties, including nano-and macroscale, crystallinity, or mechanical strength [35].

In pellets, the ratio between surface area and volume is also optimal (Figure 6).

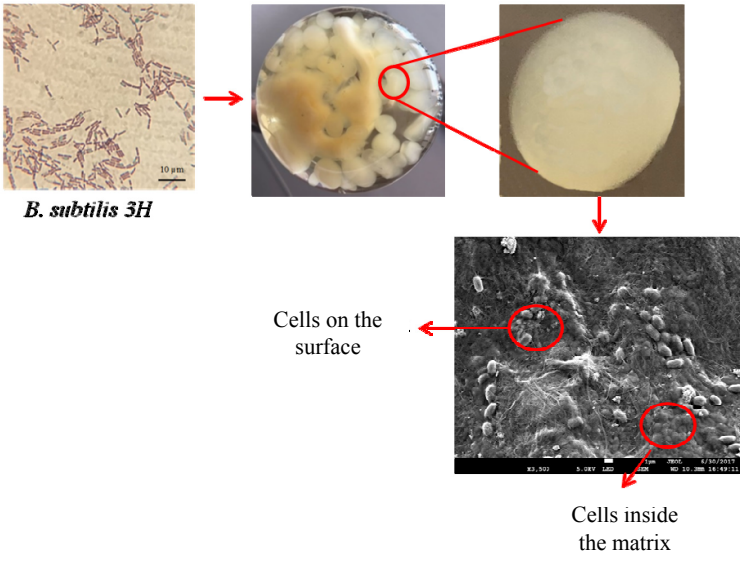


Figure 6 – Immobilization of *Bacillus* 3H bacterial cells in BC granules

Probiotic microorganisms enter the cellulose granules. Moreover, more bacterial cells were concentrated in the middle of the globules, while only individual *Bacillus* cells were found on its surface. The total number of bacterial cells immobilized by this method is 8×10^{10} CFU/g. After freeze-drying, the bacterial titer decreased by 2 orders of magnitude. Ready-to-use BC microgranules contained 10^8 CFU/g of viable *Bacillus* 3H.

3.2 Determination of the antimicrobial and probiotic activity of an experimental sample of a biocomposite

Antimicrobial activity is the most important characteristic of the effectiveness of probiotic microorganisms, so it seemed appropriate to determine this indicator *in vitro* conditions.

Probiotics based on bacteria of the *Bacillus* genus are “self-eliminating antagonists”, i.e. their main purpose is to suppress intestinal pathogens. Their etiological cause is microorganisms such as Salmonella and colibacteria. In this regard, clinical isolates of *Salmonella typhi* (PM1, PM2, PM3a), *Salmonella typhimurium* (W1, W4, W5, PM1a, PM2a), *Citrobacter aerogenes* (PM1, PM2), and *Escherihia coli (W3, W4), Proteus vulgaris* (PM1, PM2) were used as target microorganisms to study the antimicrobial activity of an immobilized spore probiotic. In this series of experiments, the antimicrobial effect was determined after co-culturing target microorganisms with BC/*Bacillus* granules in nutrient broth for 24 and 48 hours with cells of test strains. 1 g of the preparation was added to the system. They were added to the suspension of test strains (10^8 cells/ml) in nutrient broth. The inhibitory effect of the preparation was determined by the percentage of surviving cells of test strains (Table 1).

The BC/*Bacillus* biocomposite suppresses the growth of test strains up to 85.4%, i.e. the cell survival rate after 24 hours of contact with the biocomposite is on average 14.6%. After 48 hours of cultivation with the biocomposite, only 1.5% of cells remain alive. According to the requirements of Pharmacopoeial articles on probiotics, the number of live cells of test strains after 72 hours of co-cultivation with probiotic bacteria should not exceed the level of 2 % compared to the control [8-9]. An immobilized spore probiotic has a similar effect after 48 hours of co-cultivation with target microorganisms. After 72 hours, the biocomposite completely suppresses the growth and viability of these test organisms *in vitro*.

Table 1 – Antibacterial activity of BC/Bacillus biocomposite

Targets	% survival rate of test organisms	
	Contact time	
	24 hours	48 hours
E. coli W3	9	0.6
E.. coli W4	11	0.8
E. coli PM1	13	1.1
E. coli PM2	19	1.9
S. typhi PM1	14	1.4
S. typhi PM2	13	0.9
S. typhi PM3a	20	1,3
S. typhimurium W1	18	2,2
S. typhimurium W4	17	2,0
S. typhimurium W5	12	1,9
S. typhimurium PM1a	10	1,2
S. typhimurium PM2a	18	1,8
Citr.aerogenes PM1	15	2,9
Citr.aerogenes PM2	12	2,1
P.vulgaris PM1	15	0,7
P.vulgaris PM2	17	1,8
Mean	14,6	1,5

The activity of probiotics in *vivo* is usually determined either by artificial infection of laboratory animals, or on models of experimental dysbiosis. Dysbacteriosis in experimental animals can be induced by the introduction of lead salts, or by using broad-spectrum antibiotics such as kanamycin, gentamicin, cefazolin. However, the most commonly used antibiotic is ampiox [36-37]. Therefore, the effectiveness of three series of experimental samples of the material was studied in outbred rats with ampiox-induced dysbiosis.

This antibiotic was administered intragastrically at a dose of 40 mg per day for 5 days. 1 g of granules with bacilli (10^8 microbial cells) was administered intragastrically for 10 days.

The laboratory animals were divided into four groups (5 animals in each):

Group 1 – animals with experimental dysbiosis (negative control);

Group 2 – animals that received BC/*Bacillus*.

Group 3 – animals that received free *Bacillus* cells (comparison group);

Group 4 – intact animals (positive control).

The intestinal microflora was studied by bacteriological methods before the introduction of the antibiotic, 5 days after its withdrawal, and at the end of the course of treatment with bacterial preparations.

Before the introduction of the antibiotic, feces of intact animals were plated on differential diagnostic media to determine the content of the main groups of indigenous bacteria in the intestines of rats.

Since a sufficient amount of information has been accumulated to date on the specific disorders in the microbiocenosis caused by ampiox [38], it made sense to determine the population level of only such microorganisms as lactobacilli, bifidobacteria, opportunistic enterobacteria, staphylococci, and yeast fungi of the *Candida* genus. These data, as well as the results obtained after determining the composition of the intestinal microflora in rats treated for 5 days with the antibiotic ampiox, are shown in Figure 7.

After completion of antibiotic therapy, opportunistic enterobacteria were detected in increased amounts in the distal part of the rat intestinal tract – up to 10^5 CFU in 1 g of feces. The concentration of hemolytic microflora and lactose-negative enterobacteria increased. Plasmocoagulating staphylococci and *Candida* fungi were detected in high titers. Otherwise, the population levels of bifidobacteria and lactobacilli increased by several orders.

Data on determining the effect of probiotics on the microbiocenosis of laboratory animals in experimental dysbiosis are shown in Table 2.

The main criteria for the bacteriological effectiveness of drug administration to experimental animals were the population level of bifidobacteria and lactobacilli, as well as qualitative and quantitative characteristics of the content of opportunistic enterobacteria, staphylococci and candida fungi in the large intestine.

Analysis of the microbial landscape of feces of laboratory animals showed that the use of granules with a probiotic (GPB) – based preparation led to normalization of the microbiocenosis of experimental animals. After administration of GPB to experimental animals, even for 5 days, the microbial profile indicators improved. It was found that microflora similar to that present in animals included in the positive control group (intact rats) is released from the intestines of rats treated with GPB. Laboratory animals treated with GPB were found to have balanced concentrations of the total amount of aerobic and anaerobic microflora and a small amount of opportunistic enterobacteria and yeast fungi, the number of which decreased by three orders of magnitude after GPB administration. At the same time, the level of content in the intestine of representatives of native microflora – bifidobacteria and lactobacilli – was restored. Moreover, when they were used, there was a rapid increase in the number of populations of bifidobacteria and lactobacilli, and their level remained even after the drug was discontinued.

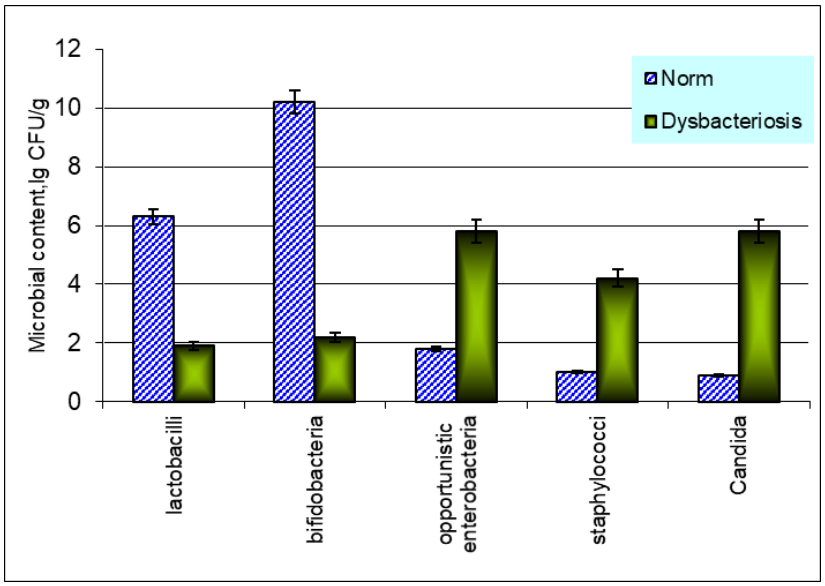


Figure 7 – Composition of intestinal microflora in animals with experimental dysbiosis

Table 2 – Composition of the intestinal microflora of rats with antibiotic-induced dysbiosis after the use of lactose-containing probiotics

Group of laboratory animals	Population level, lg CFU/g				
	Bifidobacterium	*Lactobacillus*	*Opportunistic enterobacteria*	*Candida*	*Staphylococcus*
1 – DB	5,24±0,12	4,3,35±0,24	5,54±0,22	5,86±0,22	6,81±0,08
2 – GPB	10,25±0,16	7,03±0,18	2,82±0,19	1,93±0,28	5,13±0,27
3 – FPB	8.67±0,19	7,05±0,14	3,28±0,11	3,34±0,13	5,44±0,30
4 – IA	9,82±0,81	7,62±0,19	2,25±0,22	2,34±0,14	5,27±0,19

Note: DB – animals with experimental dysbiosis; GPB – animals that received probiotic granules; FPB – animals that received free probiotic cells; IA – intact animals.
The results were significant compared to the control group at $p < 0.05$.

However, the introduction of a simple suspension of probiotic cells included in the microbiological basis of GPB did not provide such therapeutic effectiveness. It follows that the increased probiotic effectiveness of GPBs is not only due to the fact that their microbial components have an antagonistic effect, but also due to other mechanisms.

The enhanced probiotic effect of GPB can be explained by the fact that the cells of probiotic microorganisms immobilized in the BC carrier freely pass through such unfavorable upper parts of the digestive tract as the stomach and duodenum, during transit through which most of the microbial cells die.

In this regard, experiments with a " stomach model " were performed to determine the protective effect of BC on the cells attached to it. For this purpose, gastric juice (pH 1.5-2.0) was used, which was obtained during gastroscopy. It was added to a culture of bacilli in nutrient broth containing 10^8 cells/ml and incubated for one hour. Under the same conditions, 1 g of GPB was incubated, then desorption was performed, after which the number of surviving cells was determined. With such a stressful effect on the suspension of bacterial cells, their biotiter decreases by 4 orders of magnitude (Figure 8). This means that when a suspension of even a spore probiotic is administered orally, only a small fraction of their viable cells should be expected to reach the large intestine.

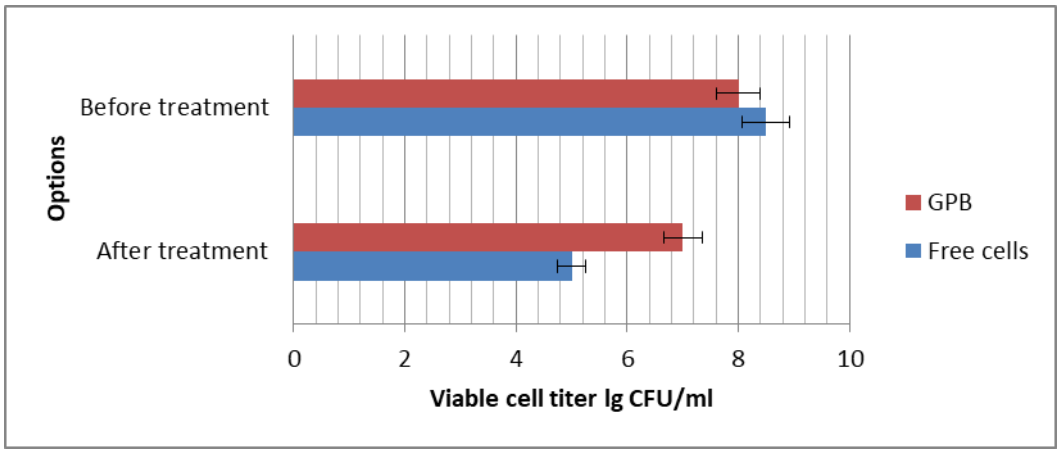


Figure 8 – Effect of an artificial gastric environment on the viability of free and immobilized *Bacillus cells*

The use of immobilized cells rather than free cells in experiments with a "model stomach" indicates their increased resistance to the bactericidal action of gastric juice. The number of viable cells in this case is reduced only by an order of magnitude. The increase in resistance observed in the experiment may be due to the fact that the cells that are part of the microcolonies formed in the sorbent are protected by a supporting matrix – BC. Therefore, immobilized probiotics significantly exceed the suspension of free cells of microorganisms in terms of resistance to the effects of gastric juice and can easily overcome the "gastric" barrier when administered orally.

This information allows us to formulate possible mechanisms of action of sorbed probiotics. It seems that their correction of intestinal microbiocenosis is carried out in several ways (Figure 9).

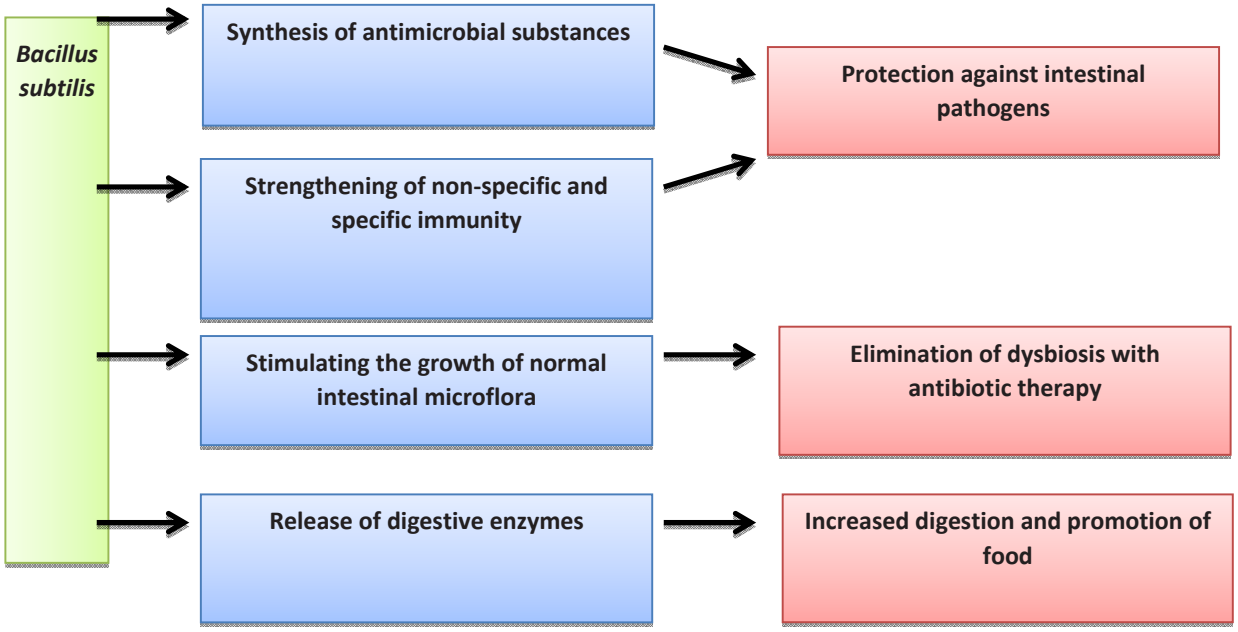


Figure 9 – Scheme of probiotic activity *of Bacillus subtilis* [14-15, 17-19, 243]

Thus, the high antibacterial effect of the BC/*Bacillus* biocomposite can be explained by the synergistic action of microbial (antagonistic, proteolytic, immunomodulatory) and protective (protection of microbial antagonist cells) components. The obtained data allow us to consider the BC/*Bacillus* biocomposite as a new sorbed probiotic for the regulation of microbiocenosis as a means of correcting intestinal dysbiosis.

Conclusion

Immobilization into globules of cells of the *Bacillus subtilis* antagonist microorganism resulted in a probiotic biocomposite containing up to 10^8 microbial cells per 1 g. The sorbed probiotic restores the intestinal normoflora of laboratory animals with antibiotic-induced experimental dysbiosis with absolute reduction and elimination of opportunistic microorganisms from the intestine. The inclusion of *Bacillus subtilis* cells in the BC matrix provides their increased resistance to the bactericidal action of gastric juice.

Conflict of interest

All authors have read and are familiar with the content of the article and have no conflict of interest.

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