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Article

New Design and Characteristics of Probiotics Immobilized on a Clinoptilolite-Containing Tuff

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Abstract: Increasing the biological effectiveness of probiotic preparations requires the development of new stable forms in the gastrointestinal tract. Live bacteria immobilized on a sorbent belong to the latest, fourth generation of probiotics, which ensures a prolonged effect. This study is devoted to developing a new method of preparing active lactobacilli on a natural mineral carrier, a tuff containing zeolite of the clinoptilolite group, which is among the most common authigenic silicate minerals that occur in sedimentary rocks and is known as a safe ion-exchange and adsorbing detoxicant. Among the characterized lactobacilli, strains of *L. plantarum*, *L. acidophilus*, and *L. crispatus* possessed a high level of acid formation and stability in gastrointestinal fluids. The protective effect of the clinoptilolite-containing tuff was registered when the samples were incubated in gastric juice. The optimal technological conditions for immobilization and lyophilization were determined, and the preservation of the viability and probiotic properties of bacteria was confirmed during 8 months of storage. The release of bacteria from the carrier occurred gradually over 12 h. The data obtained show how promising the new preparation is, combining the ability to detoxify harmful intestinal metabolites and the prolonged release of probiotics.

Keywords: probiotics; zeolite; sorption; lyophilization; survival; acid-forming activity; stability; safety; release

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1. Introduction

To restore the intestinal microbiota, a number of probiotic bacteria are currently used, mainly of the genera *Lactobacillus* and *Bifidobacterium*, the lyophilisates of which are included in capsules that partly prevent the biodegradation of probiotics in the gastrointestinal tract (GIT) and facilitate oral administration of drugs. Many brands are known, for example, Laktobalans (Cell Biotech Co., Ltd., Seoul, Republic of Korea), Bifitsin (Earth's Creation USA, Travelers Rest, SC, USA), Bak-Set forte (ADM Protexing Ltd., Somerset, UK), Bifilar (Evalar, Biysk, Russia), Normospectrum (Amphita, Moscow, Russia), etc. There are a number of problems that get in the way of the development of new forms of probiotics. In particular, the release of lyophilisates from capsules when dissolved in the GIT occurs without delay, while freeze-dried bacteria, moving through the intestinal lumen, often do not have time to transform into vegetative forms. It must be taken into account that the stage of "revival" of freeze-dried bacteria takes up to 8 h [1]; as a result, the presence of nutrient substrates for bacteria in the capsules of synbiotic preparations is ineffective because the prebiotic part is released before the probiotic bacteria can use it. It is known that the total transit time of food is 36–48 h; food stays in the stomach for 0.5–2 h, in the small intestine for 1–4 h, and in the large intestine for 30–46 h. Thus, even modern gelatin

enteric capsules, with a film remaining in the stomach for 2 h and dissolving within 0.5 h in the intestine, will ensure the massive appearance of vegetative forms from the lyophilisate only in the colon, which is densely populated with anaerobes—up to 10^{12} cells per gram of luminal contents [2]. Accordingly, colonization of the colon with probiotic strains will be difficult because of the existing microbial population of the epithelium. The alternative use of live bacteria in a non-encapsulated form on supporting media in tubes or vials limits the storage conditions and shelf life of drugs.

The above in no way detracts from the significance and prospects of using probiotics but, on the contrary, indicates the relevance of research in this developing area. In this regard, in our work, a new preparation was designed based on active probiotic strains of lactobacilli adsorbed on a mineral carrier—a tuff containing zeolite of the clinoptilolite group. We analyzed the important characteristics of immobilized lactobacilli—survival and production of organic acids during long-term preservation, as well as resistance to the fluids of the gastrointestinal tract, together with characteristics of the carrier—its structure, porosity, and cytotoxicity to eukaryotic cells in order to substantiate the correct requirements for probiotic drugs.

Clinoptilolite is a naturally occurring zeolite composed of microporous arrangements of silica and alumina tetrahedrals, linked through shared oxygen atoms, possessing a negatively charged open frame-porous structure. Neutralization of the negative charge occurs by cations contained in the zeolite (Na^+, K^+, Mg^{+2}, Ca^{+2}), capable of ion exchange. Spherical clinoptilolite is less toxic compared with fibrous and lamellar zeolites [3–5] and is used as an ion exchanger and sorbent for the needs of industry and agriculture, for the purification of contaminants, and for detoxification in veterinary and medicine [6,7]. We previously established that the release of loaded protein from clinoptilolites occurs gradually throughout the day [8], which gives grounds to assume a prolonged release of lactobacilli from this carrier. The objectives of this work included culturing a number of known probiotic lactobacilli from various sources, confirming their taxonomic identity, and developing a protocol for their sorption on the clinoptilolite-containing tuffs (CCTs) followed by freeze-drying. At the same time, the properties of CCTs, which determine the efficiency of sorption and the safety of the carrier, were estimated. The final objectives were to analyze the dynamics of the viability and release of lyophilized strains over a long period and confirm the preservation of their probiotic properties.

2. Materials and Methods

2.1. Bacteria and Their Cultivation

This study included the following 5 strains from different commercial preparations: *Lactobacillus plantarum* B-11007, VKPM, new taxonomic name *Lactiplantibacillus plantarum* (Lactobacterin dry, Biomed, Moscow, Russia), *Lactobacillus delbrueckii* ssp. *bulgaricus* Hansen Lb-12 (Danisco, France), *Lactobacillus acidophilus* B-7747, VKPM (BakZdrav, Moscow, Russia), *Lactobacillus fermentum* Lyofast LF 55 (Sacco, Cadorago, Italy), and *Lactobacillus crispatus* LMG 9479 (Ecofemin Floravag, Belgium). The taxonomic affiliation of the strains was confirmed by time-of-flight mass spectrometry with matrix-assisted laser desorption/ionization MALDI-TOF MS (mass spectrometer MALDI-7090 with the MALDI Solutions^{TM} software package, Shimadzu, Kyoto, Japan). Cultivation and preservation of lactobacilli was carried out at 37 °C using liquid and agar (2%) MRS media of the following composition (g/L): yeast extract—4.0, meat extract—10.0, casein hydrolyzate—10.0, glucose—20.0, ammonium citrate—2.0, acetate sodium—5.0, KH_2PO_4—2.0, $MgSO_4 \times 7H_2O$—0.2, $MnSO_4 \times 4H_2O$—0.05, and pH 6.2–6.5.

2.2. Mineral Carriers

CCTs from the first unit of the Tatarsko-Shatrashan deposit (Drozhanovsky district of the Tatarstan Republic) from a depth of 7–25 m were used as mineral carriers. The deposit contains marl-siliceous sedimentary rocks represented by clinoptilolite $(K_2Na_2Ca_2)_3[Al_6Si_{30}O_{72}] \times 20H_2O$), which is the least toxic compared with other zeolites [3–5]. The chemical composition of CCT is uniform (%), containing SiO_2—52.87, Al_2O_3—6.23, Fe_2O_3—2.48, CaO—15.88,

MgO—1.46, Na_2O—0.14, K_2O—1.18, P_2O_5—0.1, and loss on ignition ($H_2O + CO_2$)—19.73. The total cation exchange capacity is high—88.42 mg-eq/100 g—and the main share falls on the calcium cation Ca^{2+}—7.40 mg-eq/100 g. The following participation of other cations in the exchange is of subordinate importance (mg-eq/100 g): K^+—6.55, Na^+—1.74, and Mg^{2+}—2.73 [8]. Technological heat treatment for 6 min at 1250 °C (muffle furnace EKPS 50 model 5007, Smolensk SKTB SPL, Smolensk, Russia) was carried out to eliminate residual organics. Clinoptilolite was ground using a mechanical mill to the specified particle sizes. The samples were numbered based on the particle diameter as follows: No 1 (up to 40 μm), No 2 (40–60 μm), and No 3 (0.2–0.8 mm).

2.3. Immobilization of Bacteria

For immobilization, the Djukić–Vuković technique with some modifications was used [9]. Lactobacilli were cultivated in MRS medium under microaerophilic conditions, a 16 h culture in a volume of 200 mL was subjected to centrifugation at 10,000 rpm for 5 min, and the sediment was washed twice with sterile 0.9% NaCl and resuspended in 200 mL of fresh MRS medium with the addition of the appropriate CCT (1–5% of the total volume in different versions). The amount of lactobacilli in the suspension averaged 5.7–5.9 $\times$ 10^8 CFU/mL. The suspension was incubated at 37 °C on a shaker (180 rpm, 20 h) and then centrifuged (1000 rpm, 5 min). The supernatant containing non-immobilized cells was removed; the sediment was washed twice with phosphate-buffered saline (0.01 M, pH 7.4) and used for lyophilization. The immobilization coefficient, reflecting the sorption capacity of CCTs, was calculated as the ratio between the number of viable cells (CFU/mL) in the suspension before immobilization and in the supernatant after immobilization, centrifugation, and washing twice with phosphate-buffered saline. The immobilization coefficient was expressed as a percentage, taking the initial number of lactobacilli in the suspension as 100%.

2.4. Lyophilization

After immobilization of the biomass on CCT samples, the supernatant with unbound cells was removed and a lyoprotective sucrose–gelatin–milk medium (SGM) was introduced under aseptic conditions as follows (g/L): sucrose—100.0, gelatin—15.0, and skimmed milk powder—60. Each bacterial pellet, containing 100 mg of the corresponding CCT sample, was resuspended in 0.5 mL of lyoprotective medium and packaged in glass ampoules using a polypropylene catheter. Non-immobilized lactobacilli in equivalent quantities were used as control samples. The necks of the ampoules were closed with sterile aluminum caps and frozen at −70 °C, after which they were subjected to dehydration for 5 h using a FreeZone Dryer (Labconco Corporation, Kansas City, MO, USA) at a vacuum depth of 0.035 torr. Then, the quality of the lyophilisates in ampoules was assessed according to the following criteria: color (uniform, the color of the CCT), volume (not reduced compared to the original), the presence of bubbles/liquid in the lyophilic mass (none), large porosity (none), and the mass peeling off from the walls of ampoules upon dissolution (fast, uniform). Ampoules with samples that met the criteria were sealed under vacuum and stored for long-term preservation at (4 $\pm$ 2) °C.

2.5. Transmission Electron Microscopy and X-ray Microtomography

For the visualization of lactobacilli-loaded CCT, a drop of each sample was placed on a carbon-coated grid and, after ethanol evaporation, analyzed using a Hitachi HT7700 Exalens transmission electron microscope (Hitachi High-Tech Science Corporation, Minato-ku, Japan) at 20 kV and a resolution of 1.4 Å. Bright-field images were acquired at an accelerating voltage of 100 kV using an AMT XR-81 camera. To obtain two-dimensional images of the carriers, a General Electric V | tome | X S 240 microfocus research X-ray system for computed tomography (GE Deutschland, Frankfurt am Main, Germany) was used at a voltage of 100 kV, a current of 100 mA, and a shooting resolution for samples of 2 μm. Images were analyzed using the Avizo computer program. Two-dimensional images are

represented by orthogonal slices taken along one of the sample planes. Porous spaces were visualized in blue. For each sample, the following values were determined: intrinsic volume (mm^3), pore volume (mm^3), pore fraction (%), and diameter (μm).

2.6. Probiotic Properties of Immobilized Bacteria

2.6.1. Viability

Determination of the number of viable lactobacilli was carried out by the method of serial dilutions, where 100 μL of microbial suspensions were sown on MRS agar to determine the number of CFU. Colony counts were carried out after 48 h of incubation at 37 °C. The ampoules with lyophilisates were sterilely opened and restored to the original volume with an isotonic NaCl solution (including incubation for 10 min at room temperature with stirring to dissolve flakes and lumps completely). Next, serial dilutions were performed in liquid MRS medium and plated on MRS agar; after 48 h of incubation, the number of CFU/mL was assessed. The procedure was repeated monthly.

To estimate the prolonged release of bacteria from the carrier during 12 h, colony counts were carried out from lyophilisates without stirring.

2.6.2. Acid-Forming Activity of Lactobacilli

The amount of organic acids (lactic acid in the case of obligate homofermentative species) formed by lactobacilli was determined by the acid–base titration method. For this purpose, an overnight culture of lactobacilli was added to fresh MRS medium at a ratio of 1:100 and incubated microaerophilically for 48 h at 37 °C. The acid formation activity in each test tube was determined by titrating a 10 mL sample with 0.1 M sodium hydroxide solution until pH 8.5 ± 0.1 was achieved. The indicator of acid formation activity, expressed in Turner degrees (T) [10], was calculated using the following formula:

$$T = A \times K \times 10,$$

where

A—volume of 0.1 M sodium hydroxide solution used to titrate 10 mL of the suspension studied, mL;

K—correction factor to the titer of 0.1 M sodium hydroxide solution;

The number 10—the volume of the analyzed sample, mL.

2.6.3. Resistance of Bacteria in the Gastrointestinal Tract

We used the following model environments that simulate the conditions of the human GIT:

(a) Sublimated gastric juice of the following composition (g/L): peptone—8.3, glucose—3.5, NaCl—2.05, KH_2PO_4—0.6, $CaCl_2$—0.11, KCl—0.37, canned medical bile (Samson-Med LLC, St. Petersburg, Russia)—0.05, lysozyme—0.1, and pepsin—0.0133, pH 2.5 [11];

(b) Freeze-dried intestinal juice of the following composition (g/L): KCl—0.3, NaCl—8.0, KH_2PO_4—0.25, and Na_2HPO_4—1.44, pH 6 [12].

In test samples, 0.1 mL of rehydrated lactobacilli was added to 1 mL of gastric or intestinal juice; in control samples, 0.1 mL of rehydrated lactobacilli was added to 1 mL of nutrient medium. The samples were incubated at 37 °C for 2 h to simulate gastric stress conditions, and for 6 h to simulate intestinal stress conditions. After incubation, a series of dilutions was carried out from 10^{-2} to 10^{-10} in MRS medium, sown by the anaerobic plate count method on MRS agar plates and incubated at 37 °C for 48 h. The number of viable bacterial cells was determined by counting the colonies. The degree of resistance to gastric or intestinal stress (RD—Resistance Degree) was determined using the following formula:

$$RD = n1/n2,$$

where

n1—number of CFU/mL in the control sample;
n2—number of CFU/mL in the test sample.

Resistance to gastric and intestinal stress was assessed according to the scale of Pinto et al. [13] as follows:

Very good: RD $\leq$ 5;
Good: 5 < RD $\leq$ 10;
Acceptable: 10 < RD $\leq$ 15;
Unacceptable: 15 < RD.

2.7. Cytotoxicity of the Carriers

Assessment of human lung adenocarcinoma A549 cell and cow embryonic lung epithelium LEK (Russian Collection of Vertebrate Cell Cultures) viability in the presence of CCT extracts was carried out in accordance with the state regulatory document [14]. Phosphate buffer (0.01 M, pH 7.4) (PanEko, Moscow, Russia) was chosen as an extraction medium. Sterile CCT samples (1 g) were added to 5 mL of buffer in 15 mL centrifuge tubes (SPL Life Sciences, Pocheon-si, Republic of Korea) and incubated at 37 °C for 24 h or for 7 days. Then, the samples were centrifuged for 10 min at 4000 rpm (Eppendorf 5702R, Helicon., Moscow, Russia), and the supernatant was collected and filtered through a filter (Corning, Corning, NY, USA) with a pore diameter of 0.22 μm. Extract samples were stored at 4 °C under sterile conditions. The cells were cultured in DMEM medium (PanEco, Moscow, Russia) supplemented with 2 mM glutamine (PanEco, Russia), fetal calf serum (Biosera, Cholet, France), and 100 U/mL penicillin and streptomycin (PanEco, Russia) in a humidified atmosphere with 5% CO_2. The cells were seeded into adhesive 96-well plates (SPL Life Sciences, Republic of Korea) in an amount of 1×10^4 cells/well and incubated for 24 h; then, the medium in each well was replaced with a fresh one with the addition of the test extracts to the medium in ratios of 1:4; 1:8; 1:16; 1:32; and 1:64. A DMEM medium with the addition of an appropriate amount of sterile phosphate buffer served as control. The cells were incubated in the presence of CCT extracts for 48 h, and viability was assessed using the MTT test [15]. At the end of the incubation period, the medium was aspirated from each well and replaced with a medium containing tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) at a final concentration of 0.5 mg/mL. The cells were incubated for 2 h at 37 °C, and the presence of formazan crystals in the wells was visually determined using microscopy. Formazan crystals from the aspirated medium were dissolved with dimethyl sulfoxide (DMSO, Tathimfarmpreparat, Kazan, Russia), added in an amount of 100 μL/well, and incubated in the dark at 25 °C for 10 min. The optical density of the solutions was measured at 570 nm (reference wavelength 630 nm) using a BioRad xMarkTM Microplate Spectrophotometer (Hercules, CA, USA). Cell viability was calculated in arbitrary units as the ratio of the optical density of the formazan solution in the options with extracts to the density of corresponding variants with buffer. For each option studied, three biological replicates of five technical replicates were performed.

2.8. Statistics

The obtained data were processed by methods of variational statistics. The statistical significance of differences among groups was assessed using the nonparametric Mann–Whitney test. Differences were considered statistically significant at $p \leq 0.05$. The results are presented as the arithmetic mean and its standard deviation (M $\pm$ m).

3. Results

3.1. Determination of Optimal Technological Protocol for the Immobilization of Lactobacilli on Mineral Carriers

One of the key stages in the design of immobilized probiotics is the selection of a suitable carrier, the properties of which determine the degree of protection of microorganisms from the aggressive effects of biological fluids, as well as the ability to be released

directly into the GIT. The main requirements for carriers used in the industrial production of probiotics are high biological stability, sorption capacity, sufficient permeability for substrates, porosity, lack of toxicity, availability, and cost-effectiveness. High sorption capacity is a necessary technological property for tested carriers and one of the main criteria when determining the suitability of a carrier for preparing a probiotic.

The assessment of the completeness of immobilization revealed that the optimal concentrations of the carriers to avoid phase separation of the suspension and effectively bind at least 5.5×10^8 CFU per 1 g of sorbent are in the range of 1.5 to 2.5%. Based on this, further work was carried out with 2% suspensions of CCTs. The data in Table 1 show that when cells were immobilized on CCTs No 1 and No 2 (with particle diameters up to 60 μm inclusive), the completeness of binding in all cases was more than 93%, while for samples of bacterial suspensions adsorbed on CCT No. 3, it was of a significantly lower ratio, and the samples themselves showed phase separation when incubated for 4 h. It was also noted that in most control samples containing unbound lactobacilli, the number of CFU after incubation was 1–2 orders of magnitude less than in samples with immobilized lactobacilli (Table 1). The results obtained indicate the effectiveness of immobilization on carriers with a smaller particle diameter, which is associated with a larger surface area. Sample No 3 in this case, because of its lower sorption capacity and instability, does not meet the requirements for carriers used in the design of immobilized probiotics.

Table 1. Completeness of lactobacilli binding to clinoptilolite-containing tuff.

Lactobacilli	CCT No	Non-Immobilized Bacteria, CFU/mL		Immobilization Coefficient, % *
		Before Immobilization	After Immobilization	
L. plantarum	1	5.7×10^8	1.40×10^6	97.51 ± 4.7
	2	5.7×10^8	3.40×10^7	94.2 ± 9.5
	3	5.7×10^8	7.90×10^7	86.2 ± 8.5
L. fermentum	1	5.7×10^8	7.58×10^6	98.67 ± 5.12
	2	5.7×10^8	3.80×10^7	93.3 ± 6.13
	3	5.7×10^8	1.49×10^8	73.7 ± 5.3
L. delbrueckii ssp. *bulgaricus*	1	5.8×10^8	1.97×10^7	96.6 ± 5.4
	2	5.8×10^8	2.95×10^7	94.9 ± 2.3
	3	5.8×10^8	6.90×10^7	88.1 ± 6.7
L. acidophilus	1	5.7×10^8	1.10×10^7	98.1 ± 1.4
	2	5.7×10^8	1.20×10^7	97.9 ± 3.7
	3	5.7×10^8	5.01×10^7	91.2 ± 6.2
L. crispatus	1	5.9×10^8	1.08×10^7	98.16 ± 3.4
	2	5.9×10^8	3.30×10^7	94.5 ± 2.2
	3	5.9×10^8	1.23×10^8	79.2 ± 5.6

* Mean values (M) for n = 3 are presented. The number of bacteria before immobilization was taken for 100%.

3.2. Effect of Immobilization on the Stability of Lyophilized Cultures

Of the many technologically available methods for stabilizing moisture-containing materials of biological origin, the most widely used in the production of probiotics is lyophilization—freeze-drying under vacuum—which allows for preserving their viability and biological activity during the regulated shelf life of the drug. In lyophilized cultures, a significant part of the bacteria remains viable for many years without losing their probiotic properties. When optimizing the conditions for the lyophilization of bacterial cultures, it is important to select and adapt the sublimation and drying modes to the technical capabilities of a specific drying unit.

To study the protective and structure-forming properties of the carriers, freeze-drying of immobilized lactobacilli was carried out using a lyoprotector generally accepted in the production of probiotics—SFM medium. A suspension of non-immobilized lactobacilli

served as a control. When lyophilizing samples, we used a freeze-drying regime identical to that used in the production of bifidum- and lactobacterin [16]; the volume of the frozen layer in different samples varied from 0.1 to 1.0 mL. Analysis of lyophilized biomass, which was lactobacilli immobilized on CCTs No 1 and No 2, showed that the optimal volume of the frozen layer is 0.5 mL.

Ampoules with samples of volumes from 0.1 to 0.4 mL contained overdried biomass with impaired integrity; samples of volumes from 0.7 to 1.0 mL required an increase in lyophilization time to 7 h. At least 94% of the samples with a volume of 0.5 mL were dry homogeneous amorphous masses of light-yellow color with shades of the color of the samples, the mass lagging behind the walls of the ampoules occurred quickly and evenly, and the average residual moisture content in the studied samples was 0.4 ± 0.02%. The physical properties of the samples containing non-immobilized lactobacilli did not differ from the properties of the experimental samples. Transmission microscope images of immobilized lactobacilli indicate the distribution of zeolite-associated bacteria on the particles (Figure 1).

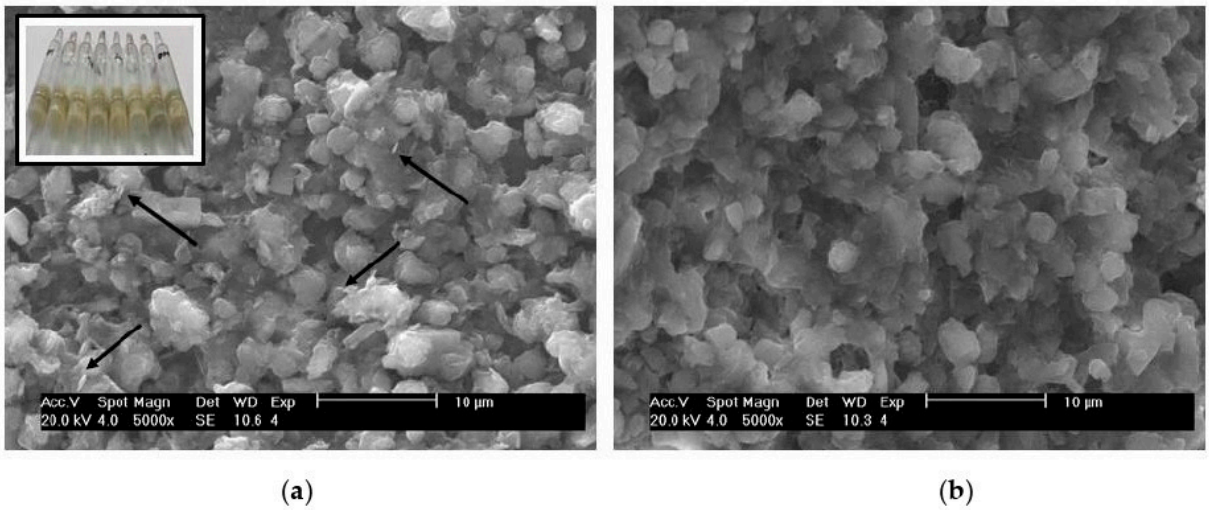

Figure 1. TEM images of clinoptilolite-containing tuff loaded with lactobacilli marked with arrows and appearance of the resulting preparation (**a**); unloaded clinoptilolite (**b**).

Thus, it was shown that the applied sublimation mode makes it possible to obtain a dry preparation whose physical properties (appearance, macrostructure, rehydration time) satisfy the conditions of commercial preparations. Lyophilisates that met the above-mentioned criteria were stored at 4 ± 2 °C. Periodic monitoring of survival, as well as the stability of probiotic properties, was carried out monthly for 8 months.

The applied lyophilization regime and the composition of the lyoprotective medium made it possible to achieve the initial viability of lactobacilli mostly at a level of at least 80% of their initial number (Table 2). During periodic monitoring of viability, it was revealed that in all the samples of lyophilized lactobacilli, a slight decrease in the number of CFU was recorded—by the end of 3 months of storage, the lyophilisates lost on average up to 10–13% of viable cells compared with the initial values. Throughout the entire storage period of the lyophilisates, their macrostructure showed no signs of external degradation. No statistically significant differences were recorded between the experimental and control samples, based on which we can conclude that the presence of carriers in the samples does not have a negative effect.

During the storage of bacterial cultures, changes in their physiological and morphological characteristics are possible because of their rehydration. Such phenomena can lead to changes in probiotic properties, the most significant of which are the ability to synthesize lactic acid and the resistance of bacteria to the stress effects in the GIT. The dynamics of

changes in the probiotic properties of immobilized bacteria were monitored after 2, 4, 6, and 8 months of storage.

Table 2. Survival of lyophilized forms of immobilized lactobacilli during long-term preservation.

Lactobacilli	CCT No	Viable Bacterial Cells, % *									
Time, Month		0	0.5	1	2	3	4	5	6	7	8
L. plantarum	1	81.8	79.4	77.1	72.9	69.9	69.4	68.1	67.2	65.1	64.9
	2	80.7	77.9	77.0	75.4	75.3	73.9	70.1	69.6	68.3	65.5
	C	80.9	83.8	83.4	82.1	80.9	80.3	78.5	74.7	72.9	70.8
L. fermentum	1	78.8	78.5	77.9	76.1	75.5	75.1	74.9	73.3	70.2	68.2
	2	76.2	75.9	75.5	74.1	72.4	70.9	70.1	68.8	65.9	63.5
	C	79.2	79.1	78.5	76.3	74.8	74.0	73.1	71.6	70.1	68.5
L. delbrueckii ssp. *bulgaricus*	1	84.3	83.9	81.2	80.7	78.8	75.4	73.8	72.1	72.0	70.9
	2	81.9	81.2	80.4	78.7	76.5	73.2	71.8	70.2	69.7	68.2
	C	83.3	82.1	79.9	79.5	78.6	78.2	72.4	71.9	70.4	67.9
L. acidophilus	1	84.5	84.5	82.1	80.9	77.3	74.2	73.9	73.5	70.1	64.9
	2	82.4	81.8	80.9	80.0	79.7	75.4	72.9	70.8	70.2	68.1
	C	81.9	81.5	81.1	80.3	79.4	78.2	75.4	73.1	72.9	70.7
L. crispatus	1	82.2	80.1	78.7	76.5	75.3	73.2	71.9	70.2	69.9	65.1
	2	83.9	82.8	80.1	79.3	78.1	76.5	74.3	72.1	70.1	69.3
	C	80.2	78.3	78.1	77.8	74.3	72.1	71.9	70.2	69.2	68.9

* Mean values (M) for n = 3 are presented; C—control, lyophilisate without immobilization. The initial number of bacteria before immobilization was taken for 100%.

3.3. Acid-Forming Activity of Immobilized Lactobacilli

It is well known that the inhibition of pathogen growth and reproduction is determined by the spectrum of acids produced by probiotic bacteria [17]. The antimicrobial effect of lactic acid has been studied in detail, and the activity of acid formation is considered to be an indicator of the specific activity of lactobacteria-containing probiotics and, as a consequence, one of the criteria for selecting test strains for the development of probiotic preparations. The dynamics of the changes in the acid-forming activity of lactobacilli immobilized on clinoptilolite are shown in Table 3.

Table 3. Dynamics of the changes in acid-forming activity of immobilized lactobacilli.

Lactobacilli	CCT No	Acid-Forming Activity, T *				
		Preservation Time, Month				
		0	2	4	6	8
L. plantarum	1	219.2	217.9	215.6	211.8	207.4
	2	213.4	210.7	208.4	206.5	203.1
	C	216.9	212.4	209.2	207.6	205.1
L. fermentum	1	198.4	196.1	195.9	193.3	190.9
	2	201.2	198.2	196.7	195.0	194.6
	C	205.1	202.7	199.4	196.6	191.0
L. delbrueckii ssp. *bulgaricus*	1	189.7	186.6	182.9	180.7	177.4
	2	196.2	195.9	193.1	190.9	188.6
	C	192.2	189.4	187.1	182.2	177.3
L. acidophilus	1	243.7	239.9	236.4	231.1	229.8
	2	245.8	241.7	238.8	236.4	231.1
	C	241.1	237.8	233.4	227.8	226.5
L. crispatus	1	234.3	231.4	226.4	220.1	217.7
	2	237.7	234.5	231.9	226.4	221.4
	C	232.2	230.7	229.6	225.3	220.9

* Mean values (M) for n = 3 are presented; C—control, lyophilisate without immobilization. Standard deviation was less than 3%.

Throughout the entire storage period of the lyophilisates, the indicator of acid formation activity decreased slightly and, in most of the studied strains, did not fall below

170T at the end of the storage period, which is a satisfactory indicator for lactobacilli when cultivated on MRS medium. According to the test, the most promising of the studied strains were *L. acidophilus* and *L. crispatus*, which are strong acid-producers. The data obtained allowed us to state that the immobilization of lactobacilli on the carrier does not affect the level of acid-forming activity, which contributes to their antagonistic properties.

3.4. Resistance of Immobilized Lactobacilli to Gastrointestinal Stress

The stability of the tested lactobacilli strains under conditions of gastrointestinal stress is an important probiotic characteristic [18]. Some members of the genus Lactobacillus are considered highly acid-resistant [19]; however, despite species and strain differences, most of them are sensitive to pH values below 3.0 [20].

It is known that the resistance of probiotic microorganisms to the action of the human GIT media increases when they are immobilized on certain carriers. The results of assessing the stability of lyophilisates at different storage periods showed that immobilized lactobacilli tolerated the action of freeze-dried gastric juice well for 2 h: the RD value was no more than 8.36, while in the non-immobilized samples, the resistance potential was observed to be lower (9.73) (Table 4). Based on the presented data, it is clear that the best resistance to the action of gastric juice and bile acids was demonstrated by the samples containing strains of *L. plantarum* (average RD $\leq$ 7.58), *L. acidophilus* (average RD $\leq$ 7.56), *L. crispatus* (average RD $\leq$ 7.19). No statistically significant differences were found between samples containing CCTs No 1 and No 2.

Table 4. Resistance of immobilized lactobacilli to gastric stress during long-term storage *.

Lactobacilli	CCT No	Resistance Rate, RD, Units **				
		Preservation Time, Month				
		0	2	4	6	8
L. plantarum	1	4.17	5.48	6.05	6.71	7.28
	2	4.37	5.82	6.45	7.09	7.58
	C	5.18	6.30	7.65	8.92	9.73
L. fermentum	1	4.95	5.55	7.07	8.02	8.36
	2	5.18	6.04	7.55	7.78	8.13
	C	6.12	6.86	7.09	8.92	9.58
L. delbrueckii ssp. *bulgaricus*	1	5.33	6.46	7.01	7.35	7.79
	2	5.43	5.54	6.24	6.61	7.47
	C	6.05	6.51	6.85	8.13	8.99
L. acidophilus	1	4.29	5.21	5.79	6.64	7.36
	2	4.63	5.38	6.25	6.82	7.27
	C	5.12	5.69	6.63	7.89	8.06
L. crispatus	1	4.65	5.38	5.7	6.02	7.19
	2	5.01	5.14	6.15	6.61	6.90
	C	5.56	6.57	7.84	8.46	8.56

* Mean values (M) for n = 3 are presented; C—control, lyophilisate without immobilization. ** RD—degree of resistance according to Pinto et al. [13]: "very good"—RD $\leq$ 5; "good"—5 < RD $\leq$ 10; "acceptable"—10 < RD $\leq$ 15; "unacceptable"—RD > 15.

Drugs remain in the small intestine for 4 to 6 h, which is a sufficient period to affect the functional activity of probiotics. Based on this, the stability of immobilizers under the influence of intestinal juice was studied for 6 h. Table 5 shows that the strains were initially resistant to the action of intestinal juice. All the samples of immobilized lactobacilli had a "very good" RD indicator according to the rating scale; moreover, to the end of the eighth month of lyophilisate storage in ampoules, this value did not exceed 3.46. The best survival rates were observed for the samples containing strains of *L. plantarum* (average RD $\leq$ 1.99), *L. acidophilus* (average RD $\leq$ 1.86), and *L. crispatus* (average RD $\leq$ 2.23). No statistically significant differences were detected in relation to the control samples, and no differences were recorded between samples containing CCTs No 1 and No 2. Based on this, it can be

stated that the presence of carriers in the composition of lyophilized samples does not affect the dynamics of the changes in the resistance potential of lactobacilli to intestinal stress.

Table 5. Resistance of immobilized lactobacilli to intestinal stress during long-term storage *.

Lactobacilli	Sample	Resistance Rate, RD, Units **				
		Preservation Time, Month				
		0	2	4	6	8
L. plantarum	1	1.63	1.81	1.85	1.91	1.97
	2	1.61	1.84	1.87	1.89	1.94
	C	1.72	1.87	1.88	1.89	1.99
L. fermentum	1	2.22	2.31	2.44	2.54	2.71
	2	2.26	2.33	2.49	2.59	2.63
	C	2.36	2.37	2.53	2.55	2.68
L. delbrueckii ssp. *bulgaricus*	1	2.83	2.94	3.23	3.39	3.46
	2	2.76	2.84	3.04	3.07	3.14
	C	2.62	2.68	2.75	2.96	3.09
L. acidophilus	1	1.26	1.28	1.34	1.48	1.66
	2	1.21	1.37	1.42	1.49	1.55
	C	1.35	1.43	1.47	1.69	1.86
L. crispatus	1	1.59	1.7	1.77	2.07	2.09
	2	1.54	1.61	1.66	1.82	2.17
	C	1.62	1.78	2.13	2.16	2.23

* Mean values (M) for n = 3 are presented; C—control, lyophilisate without immobilization. ** RD—degree of resistance according to Pinto et al. [13]: "very good"—RD $\leq$ 5; "good"—5 < RD $\leq$ 10; "acceptable"—10 < RD $\leq$ 15; "unacceptable"—RD > 15.

3.5. Characteristics of Carriers

Zeolites are natural minerals of more than 100 different types (clinoptilolite, chabazite, phillipsite, mordenite, etc.) with varying physical and chemical properties. When developing applications for zeolites, it is important to know that not all these minerals are the same. That is why each mineral carrier sample used for probiotic immobilization must be characterized, especially considering that usually, not zeolites themselves but zeolite-containing tuff is used.

Two- and three-dimensional images (2D and 3D) were obtained using X-ray computed microtomography (Figure 2). Two-dimensional images are represented by orthogonal slices made along one of the planes. The images visualize the porous space (in blue). For this study, an X-ray system for computed tomography was used with a shooting resolution of 2 µm for sample analysis. The error values of the method were determined by the resolution—smaller voids cannot be segmented. Image analysis using the Avizo computer program made it possible to obtain the following characteristics: the sample's intrinsic volume (0.48 mm^3), pore volume (0.038 mm^3), and the fraction of pores (7.8% from whole sample volume), as well as the equivalent pore diameter, whose distribution is shown on Figure 3. The data obtained indicate that the diameter of the pores allows bacteria, whose size is smaller than the diameter of the pores, to be adsorbed into them.

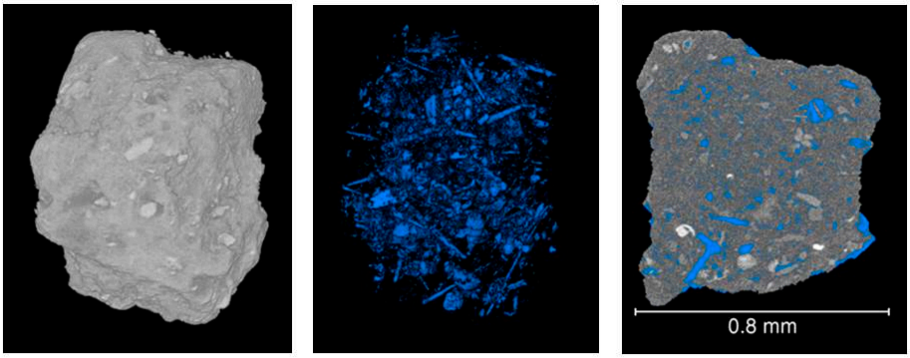

Figure 2. Characteristics of clinoptilolite-containing tuff samples from Tatarsko-Shatrashan deposit by X-ray computed microtomography: (**A**) form of a sample, (**B**) 3D image, (**C**) orthogonal slice with pores highlighted in blue color.

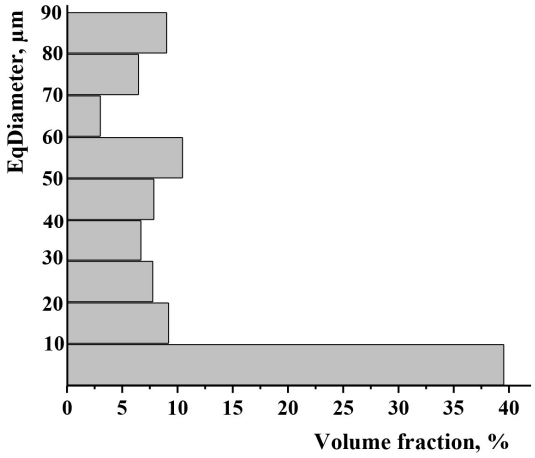

Figure 3. Volume fractions of pores according to their equivalent diameter.

3.6. Cytotoxicity of Mineral Carriers

To determine the possible toxic effects of the mineral carriers, we used their aqueous extracts according to the procedure established for solid medical devices [14]. Extracts of clinptilolite-containing tuffs did not reduce the viability of human lung adenocarcinoma A549 cells and of cow embryonic lung epithelium LEK (Figure 4). Moreover, we previously added powdered CCT itself to a monolayer of cells and showed that in this modification, the reduction in the growth of colorectal cancer Caco2 cells does not exceed 30%, while for chabazite and natrolite, this value was 40% and 70%, respectively [5]. Therefore, the mineral carrier that we used is safe.

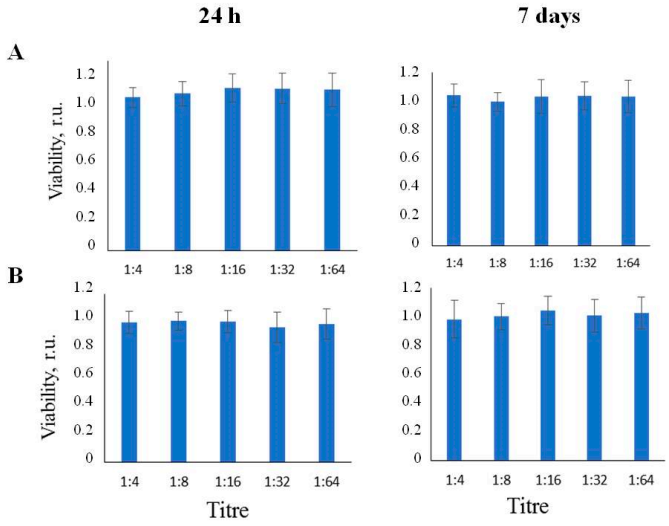

Figure 4. Changes in the viability of A549 (**A**) and LEK (**B**) cells in the presence of CCT No1 extract. The sample extract was added to the culture medium in the titer of 1:4, 1:8, 1:16, 1:32, and 1:64 to medium (*v*/*v*). The value of the viability index in the corresponding control option was taken as a relative unit equal to one.

3.7. Release of Probiotics from the Carrier

To prove that the probiotic will be released from the carrier gradually, reaching the small intestine, we modeled the release of *L. crispatus* into a physiological solution without using intense shaking and recorded the grown CFU on a solid nutrient medium. The conditional yield of the probiotic in the stomach (1 h of movement through the gastrointestinal tract) was less than 3% and within 3–6 h, almost 70% of the bacteria were released (Table 6). Within 12 h, 85% of bacteria were released from the carrier. Since the residence time of substances in the small intestine varies on average from 6 to 8 h, it can be concluded that approximately 80% of bacteria will be released in the small intestine with an increasing concentration over time. This is a very encouraging result since the lyophilized bacteria will have time to be transformed into vegetative forms and manifest their probiotic properties.

Table 6. Time-course of *L. crispatus* release from the carrier.

Release Option	Incubation Time, h	Viable Bacteria, CFU/mL	Viable Bacteria, %
Before lyophilization	0	6.0×10^8	100%
After lyophilization (stirring)	12	5.34×10^8	89.0%
After lyophilization (no stirring)	1	1.7×10^7	2.8%
After lyophilization (no stirring)	3	2.5×10^8	41.6%
After lyophilization (no stirring)	6	4.2×10^8	69.5%
After lyophilization (no stirring)	9	4.9×10^8	81.6%
After lyophilization (no stirring)	12	5.1×10^8	85.0%

4. Discussion

Modern classification of zeolites includes about 140 types of natural and 150 synthetic zeolites for specific and selective use [21]. Among the many species of zeolites, the most widespread is the naturally occurring zeolite clinoptilolite, which is a detoxifying, antioxidant, and anti-inflammatory mineral. It is used in many industrial applications ranging from environmental remediation to oral applications/supplementation for humans as food supplements or for medical devices [6]. Zeolites, because of their porous structure

and tunable properties, which can be modified with various materials, can be used as a delivery agent for theranostic applications [22], as scaffolds for tissue engineering [23], as drug delivery systems, and for wound healing. They can also be used as anti-bacterial, antimicrobial, implant-coating, and contrast agents, to remove harmful ions from the body, as gas absorbers, in hemodialysis, and as tooth root-filling material [24]. Medical applications of natural and synthetic zeolites have been intensively studied. The structure of clinoptilolite is considered to be biologically neutral and non-toxic. The results published thus far show that clinoptilolite does not affect the homeostasis of trace elements and micronutrients but rather, it acts selectively on heavy metals and toxicants. The health effects and safety in medical applications of different clinoptilolite-based materials are attributed to the restoration of human homeostasis because of local detoxification properties within the intestine, the release of dissolved silica forms from the clinoptilolite tuff that enter from the intestine into the blood, and to clinoptilolite's antioxidant and immunomodulatory effects [7]. The test we conducted confirms the safety of used clinoptilolite and is consistent with the literature. However, many publications do not contain the characteristics of the specific zeolite studied, which is most often represented not by the zeolite itself but by the zeolite-containing tuff, including purified clinoptilolite-tuff [25,26]. Rare works indicate the specific type and structure of the natural zeolites used. This fact prevents the correct interpretation of the results obtained on the adsorption of therapeutics in the pores of the carrier and their comparison with each other. The detailed description of synthetic zeolites for medical applications such as drug delivery to tumor cells happens more often [27]. The mineral assemblies of the most common zeolite occurrences in nature are clinoptilolite- and mordenite-containing tuffs, which may be accompanied by bentonite and phases present in lower percentages, such as cristoballite, calcite, feldspar, and quartz. The properties of such materials may vary in the widest sense with respect to the final mineral content [28].

Our preliminary data confirmed the effectiveness of immobilizing three strains of lactobacilli on mineral carriers with particles less than 1mm without fractionation according to average particle size [29]. In this research, we used a zeolite-containing tuff of the Tatarsko-Shatrashan deposit with different granularity. The main rock-forming components of this system are clinoptilolite, the opal–cristobalite–tridymite phase (OCT phase), clay minerals (montmorillonite), calcite, and quartz, which account for 90–95% of the tuff volume. The OCT phase and montmorillonite, along with zeolites, are natural sorbents characterized by properties (adsorption, cation exchange) that significantly complement and expand the range of physicochemical properties of tuff [30]. Zeolites have uniform pores (from 3 to 10 Å), the sizes of which are uniquely determined by the unit cage of the crystal. These pores are unable to adsorb molecules whose dimensions exceed the pore diameter. However, we characterized a zeolite-containing tuff that has pores ranging in size from clinoptilolite micropores to cavities whose size is up to 90 μm (Figures 2 and 3). The percentage of micropores in the CCT is about 40%, and the macropores available for localization of bacteria are 60% (Figure 3). This allowed us to conclude that probiotic bacteria are adsorbed not only on the surface of the carrier but can also be included in its macropores and cavities (Figure 1). Previously, we demonstrated the ability of proteins to be adsorbed on zeolite, both small cationic (RNase with a molecular weight of 12.3 kD) [5] and large anionic (albumin, 69 kD) [8]. Thus, the surface charge does not play an important role in the adsorption of biomolecules. Although in Figure 1, we see only bacteria among CCT particles and on their surface, the long-term retention of lactobacilli in the mineral indicates their localization in macropores; otherwise, the release of bacteria from the carrier would not have been prolonged, as it was established (Table 6).

We selected five known probiotic strains of lactobacilli and loaded them into clinoptilolite-containing tuffs of varying degrees of granularity: No 1 (up to 40 μm), No 2 (40–60 μm), and No 3 (0.2–0.8 mm). It was shown that immobilization on CCT with particle diameters up to 60 μm, inclusive, led to the completeness of binding, in all cases, at more than 93%, while this parameter for CCT No. 3 was significantly less (Table 1). The immobilization on carriers with a particle diameter of mm size had a significantly lower rate and did not

meet the requirements for carriers used in the design of immobilized probiotics. Then, we optimized conditions of lyophilization and maintained a high level of survival (Table 2) and organic acid production (Table 3) for 8 months of preservation. Particularly significant results on the stability of drugs were obtained when they were incubated in gastric and intestinal juices. The degree of resistance of all strains to gastric stress according to Pinto et al. [13] was estimated as "good" (RD less than 10) (Table 4); the resistance to intestinal stress was "very good" (RD less than 5) (Table 5). The absence of significant differences between the control and samples containing CCTs No 1 and No 2 indicated that carriers in the preparation support the resistance potential of lactobacilli. Thus, for the first time, we obtained stable preparations of probiotics immobilized on microgranular clinoptilolite-containing tuffs from the Tatarsko-Shatrashan deposit.

Similar publications aimed at enhancing the nutritional quality of animal feed by using a freeze-dried *Lactobacillus plantarum*–zeolite mixture, which was capable of removing up to 90% aflatoxin B1. However, the bacterial viability in this mixture decreased by one logarithmic order after 20 days of storage and three logarithmic orders after 60 days [31]. The evaluation of probiotic, nanozeolite, and/or medium-chain fatty acid effects on fish health and the chemical composition of the fish body showed that feed additives did not affect the health status of fish, as indicated by normal liver and kidney functions [32]. The combination of zeolite with probiotic bifidobacteria as a feed additive for bulls led to an increase in the weight of the animals [33]. Concerning people, a review concentrated on studies about the antimicrobial mechanisms of nanoparticles and zeolite, and it was shown that zeolites as antimicrobial components, especially in food technology and medical applications, can be considered a prominent strategy to overcome pathogenic microorganisms. The type, particle size, and shape of nanoparticles and zeolite are important factors influencing antimicrobial effectiveness [34]. One study tested the changes in anthropometric measurements, body composition, blood pressure, lipid profile, and testosterone following a low-energy-density dietary intervention including zeolite and bentonite clay and showed weight loss and improvements in total cholesterol and low-density lipoprotein cholesterol levels in voluntary participants [35]. Montmorillonite, clinoptilolite, and halloysite clays can be used to design mineral carriers not only for probiotics but also for medical substances whose immobilization, delivery, and release occur through sorption processes in the pores [36,37]. Considering modern publications, we state again that many authors did not specify whether they used zeolites or zeolite-containing tuffs. Currently, probiotic dietary supplements, including those containing zeolite, are widely advertised. These include, for example, "Litovit C" (Nov Agro, Novosibirsk, Russia) consisting of natural zeolite (or, more probably, zeolite-containing tuff), wheat bran, rye bran, bifidobacterial, and lactobacilli; "LB-complex L" (Academician I.N. Blokhina Institute, Nizhny Novgorod, Russia), a liquid probiotic, which is a consortium of live antagonistically active strains of bifidobacteria and lactobacilli immobilized on an enterosorbent zeolite; and "Bacteriostatin" (Kraft, Moscow, Russia), a metabiotic containing metabolites of *Bacillus subtilis*, a zeolite carrier, soy flour hydrolysate, and calcium stearate. Unfortunately, manufacturers do not provide detailed characteristics of the drugs, their expiration dates do not always coincide with those stated, and their effectiveness has not been proven in serious studies. A recent review of the use of zeolites and zeolite-containing tuffs indicates that the extent of bacterial immobilization does not correlate with the clinoptilolite content, cation-exchange capacity, or zeta potential, and it cannot be predicted by mineralogical and chemical analysis; therefore, each mineral sample containing different types of zeolites is a candidate for use as a carrier of bacteria provided it is non-toxic. This means that new probiotic preparations created on mineral carriers must possess a complete comprehensive characterization, and the benefits of their use must be confirmed in experiments, including studies on humans. It is known that nutrient uptake is restricted to the small intestine. Immune cell abundance at this anatomical site combined with diminished mucus secretion and looser junctions (partly to allow for more efficient fluid and nutrient absorption) also results in intimate host–microbe interactions despite more rapid transit [38]. Here, we developed a number of new combinations of

probiotic lactobacilli on a mineral carrier that remain stable, retain probiotic properties during long-term storage, and are released gradually, providing probiotic engraftment at biological sensible niches after oral administration. Further research is needed to confirm their effectiveness in animals and humans.

5. Conclusions

This work was carried out to confirm the ability of natural CCTs to serve as carriers of probiotic bacteria. Here, we characterized in detail the clinptilolite-containing tuff of the Tatarsko-Shatrashan deposit, selected the parameters of its optimal granularity (up to 60 μm) for the sorption of lactobacilli, and also showed that the sizes of the pores combined into cavities are sufficient to accommodate bacteria in them. The completeness of probiotic adsorption on the carrier reached more than 93%. The use of a lyoprotective sucrose–gelatin–milk medium after immobilization contributes to a high level of survival and organic acid production during 8 months of storage. Promising results were obtained when preparations were incubated in gastric and intestinal juices. The degree of resistance of all strains to gastric stress according to Pinto et al. [13] was estimated as "good"; resistance to intestinal stress was "very good", indicating that microgranular carriers in the preparations support the resistance potential of lactobacilli. The release of bacteria from the carrier occurs gradually over 12 h, which proves that probiotics, when administered orally, will be active upon reaching the upper intestine. The safety of carriers was proven by the absence of toxicity towards human lung adenocarcinoma A549 cells and cow embryonic lung epithelium LEK cells. Thus, the microgranular clinoptilolite-containing tuff has a high potential for practical application as a promising mineral carrier of probiotic bacteria.

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