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The liquid mineral- and probiotic-containing feed additives for poultry

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Abstract. In this study, we assessed the biocompatibility of five probiotic lactobacilli strains and characterized sapropel, bentonite, and zeolite from the deposits of Tatarstan Republic in terms of their chemical and mineral compositions, cation exchange capacities, sorption activity, and the structure of the ion exchange and sorption complexes. We also constructed a synbiotic preparation with sapropel, bentonite, and zeolite as carrier matrixes for probiotic lactobacilli and determined their viability in the preparation for two weeks. High ion-exchange and sorption properties of sapropel, bentonite, and zeolite and suitable bacterial survival rate during storage are among the main benefits of the developed poultry feed. The obtained data have a great potential for practical use in the construction of multi-strain liquid probiotics, in which probiotic lactobacilli are immobilized on mixtures of various mineral matrix carriers.

1 Introduction

In frame of the transition to organic agriculture, the development of the novel environmentally friendly probiotic feed additives is intensively developed worldwide. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. In the poultry industry, probiotics affect positively birds' gastrointestinal tract and immune system. Besides, the use of probiotics may improve the productivity of birds and quality of meat and eggs that represents an important economic benefit [2]. Probiotics are often implemented as antibiotic substitutes since the European Union and other countries have banned the use of antibiotics as growth promoters in poultry [3]. As the world's population grows, the demand for meat, eggs and other livestock products will also increase. Multi-strain probiotics are preferable because they combine the beneficial properties of several individual strains. An important property of multicomponent probiotics is the biocompatibility of starter cultures, that is, their ability to be co-cultivated. Such probiotics promote selective colonization of the intestine with the species that are most intimate to the host microbiota. Another promising approach to enhance the therapeutic effectiveness of probiotics is the creation of synbiotics, in which the beneficial

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properties of probiotic bacteria are enhanced by the valuable characteristics of other components (most often prebiotics, which stimulate the development of intestinal normal flora) [4]. Mineral natural materials, such as zeolite, bentonite, and sapropel, are viewed both as carrier matrixes for probiotics and as feed additives. Their extensive use in the poultry industry is determined by their active ion exchange complexes, sorption properties, and a wide range of macro- and microelements with beneficial biological effects in animal organisms. They also exhibit detoxification and sorption properties, exert antimicrobial activity, enhance the productivity of birds, and reduce environmental pollution [5-10]. To the best of our knowledge, complex composites based on mixtures of different minerals, as well as their complexes with probiotic bacteria or organic compounds, are extremely rare. Notably, minerals that originate from the common parent rock within one point of extraction may differ in their structure, alloy content, set of exchangeable cations, and mineral composition. Given the diversity between strains and species of probiotic microorganisms, each particular mineral- and probiotic-containing synbiotic should be subjected to a thorough investigation of its composition, safety, and effectiveness.

In this study, we assessed the biocompatibility of five probiotic lactobacilli strains and characterized sapropel, bentonite, and zeolite in terms of their chemical and mineral compositions, cation exchange capacities, sorption activity, and the structure of the ion exchange and sorption complexes. We also constructed a synbiotic preparation with sapropel, bentonite, and zeolite as carrier matrixes for probiotic lactobacilli and determined their viability in the preparation for two weeks.

2 Materials and methods

2.1 Lactobacilli strains and growth conditions

Five lactobacilli strains with earlier-described probiotic properties were used in this study. *Lactiplantibacillus plantarum* AG10 and *Lacticaseibacillus rhamnosus* AG16 were isolated from silage [11]; *Lactiplantibacillus plantarum* FCa3L was isolated from fermented cabbage [12]; *Lactiplantibacillus plantarum* LS 4-4 and *Limosilactobacillus fermentum* HFD1 were isolated from the human colon and feces, respectively [13].

All lactobacilli were grown in de Man-Rogosa-Sharpe (MRS) broth or MRS agar (HiMedia, India) at 37 °C under microaerophilic conditions unless otherwise indicated.

For biocompatibility assessment, MRS agar plates were inoculated with overnight cultures so as to obtain the intersections of perpendicular streaks. The results were assessed after 24 hours of incubation. Cultures were considered biocompatible in cases of similar or enhanced growth of the cultures in the area of co-cultivation. Cultures were referred to as bioincompatible in cases of growth retardation of one or both strains at the intersections of streaks.

The enumeration of culturable viable lactobacilli within synbiotic preparations was performed using the drop plate assay [14].

2.2 Characterization of sapropel, bentonite, and zeolite

Sapropel (Beloye Lake, Tukaevsky district, Tatarstan Republic, Russian Federation) and two agrominerals, bentonite (Tarn-Varsky deposit, Nurlatsky district, Tatarstan Republic, Russian Federation) and zeolite (Tatarsko-Shatrashansky deposit, Drozhzhanovsky district, Tatarstan Republic, Russian Federation), were used in this study. The chemical composition was determined by the method of quantitative spectral analysis on the spectrometer ES-1 as part of the diffraction spectrograph DFS-458S and the photoelectronic recording device type

FP-4. The structure and morphology of mineral particles and sapropel were studied in the intermittent contact mode by atomic force microscopy (AFM) using a MultiMode V scanning probe microscope (Veeco, USA).

2.3 Construction of a multicomponent liquid synbiotic preparation

To prepare an experimental series of synbiotic preparations, individual strains of lactobacilli were resuspended in a nutrient base of the probiotic of the following composition: 10% clarified whey, 1% yeast extract, and 0.5% sucrose. Mineral carrier matrixes (sapropel, bentonite, or zeolite) were added in a ratio of 1 g per 10^9 CFU of lactobacilli. To obtain a liquid probiotic, the required volume of the liquid phase was from 3 to 5 mL per 1 g of mineral matrix. The immobilization was performed at a temperature of + 4±1°C for 18-24 hours.

2.4 Statistical analysis

All experiments were performed in triplicate. Quantitative data are presented as the means ± standard deviations that were analyzed using GraphPad Prism 6. Statistical differences between mean values were evaluated using Mann-Whitney U test at a significance level of $P < 0.05$.

3 Results and discussion

3.1 Biocompatibility of the strains proposed for inclusion in a multi-strain preparation

We showed that five tested lactobacilli strains do not affect the growth of each other and thus can be successfully used in a multi-strain preparation.

3.2 Characterization of sapropel, bentonite, and zeolite proposed as carrier matrixes in a multi-component preparation

Table 1 presents a comparative description of the chemical and mineral compositions, cation exchange capacities, sorption activity, and the structure of the ion exchange and sorption complexes of sapropel, bentonite, and zeolite.

Sapropel represents ancient bottom sediments of freshwater reservoirs, which were formed from the remains of living organisms, plankton, dead aquatic vegetation, and particles of soil humus, and thus contains a large amount of organic substances and microelements. In its natural state, sapropel is a gelatinous or pasty mass, greasy to the touch, with a water content of 70-75%. In the composition of sapropel, silt solution, skeleton, and colloidal complex are differentiated. The silt solution contains water with dissolved minerals, low-molecular-weight organic compounds, amino acids, vitamins, and enzymes. The skeleton is the undecomposed remains of plant and animal origin, and the colloidal complex is complex organic substances that give sapropel a jelly-like consistency [15]. The extraction of sapropel is a labor-intensive process associated with the lifting of silt masses from the bottom of lakes, their drying and processing. During processing, sapropel is dried to 7-8% moisture.

Bentonite clay is an agromineral with the general formula:

$$(Ca, Na)\ (Mg, Al, Fe)_2\ (OH)_2\ [(Si, Al)_4\ O_{10}] \times nH_2O$$

In its natural state, bentonite is dense, sticky, and viscous, ranging from green-blue to yellow-brown in color. Bentonite clay is subjected to thermal-mechanical activation (900 °C for 1 hour) to obtain a bentonite powder with a particle size of 0.18 mm.

Zeolite is an aqueous framework aluminosilicate of alkali and alkaline earth metals with the general formula:

$$M_{x/n}[(Al_2)_x(SiO_2)_y] \times zH_2O$$

where M are cations with valency n, z is the number of water molecules, and the y:x ratio ranges from 1 to 5 [16]. For practical use, zeolites are exposed to mechanical activation (mechanical grinding), and a fractionated zeolite with particles ranging in size from 0.02 to 7 mm is obtained.

The agrominerals have different ion exchange, catalytic, sorption, and abrasive properties, which significantly depend on their structure, chemical composition, and mineral composition.

The main active ingredient of bentonite clays is montmorillonite, which determines the color and ion exchange, catalytic, and sorption properties of the agromineral. In terms of montmorillonite content, bentonite is superior to the other studied agrominerals. The advantage of zeolites is that they contain opalcristabolite, calcite, and clinoptilolite, which give the agromineral a fixed lattice structure. Undesirable components in agrominerals are quartz, hydromica, and kaolinite.

The sorption properties of agrominerals are maintained by silica SiO_2 and alumina Al_2O_3. The total amount of SiO_2 and Al_2O_3 in bentonite was 1.3 times higher than that of zeolite and 4.6 times higher than that of sapropel. Consistently, the cation exchange capacity of bentonite exceeded that of zeolite and bentonite, but sorption was highest for zeolite (Table 1). The sorption and ion exchange properties of zeolite originate from its particularly strong crystal lattice. Benthic powder is characterized by a mobile belt-layer structure with swelling packets, which provides ion exchange and sorption activities.

The structure of sapropel is determined by the structure of the mineral rocks included in its composition and suggests the presence of various sorption surfaces, from a fixed crystal lattice and a belt-layered structure to an amorphous one, represented by parts of the skeletons of plants, protozoa, and others. The organic part of sapropel is represented by low-molecular-weight proteins, enzymes, amino acids, vitamins, etc., which significantly reduce the sorption properties. Biogenic elements are also represented by inclusions of gibbsite, saponite, halloysite, and chlorite. The mineral part of sapropels is very variable. Along with a high amount of iron (II, III) oxides and calcium oxides, sapropel contains kaolinite, montmorillonite, vermiculite, and amorphous silica that provide various ion exchange and sorption properties.

In the sapropel, the following amino acids were determined as presented in descending order of their content: glycine, aspartic acid, serine, glutamic acid, tryptophan, leucine, lysine, valine, alanine, phenylalanine, isoleucine, tyrosine, arginine, threonine, cystine, methionine, and histidine.

A toxicological and microbiological assessment of the sapropel from Beloye Lake stated the absence of cadmium, tin, arsenic, radionuclides, and pesticides, as well as pathogenic biological agents.

In terms of sorption properties, the studied carrier matrixes can be arranged in the following descending order: zeolite ≥ bentonite > sapropel. Based on the sum of exchangeable cations that provide the ion-exchange properties, the descending series is as follows: bentonite > zeolite > sapropel. Thus, the studied agrominerals and sapropel possess high ion-exchange and sorption properties and are suitable for the incorporation of lactobacilli cells into their spatial matrix.

Table 1. Characterization of sapropel, bentonite, and zeolite proposed as carrier matrixes in a multi-component preparation.

	Bentonite	**Zeolite**	**Sapropel**
Point of extraction	Tarn-Varsky deposit, Nurlatsky district, Tatarstan Republic, Russian Federation	Tatarsko-Shatrashansky deposit, Drozhzhanovsky district, Tatarstan Republic, Russian Federation	Beloye Lake, Tukaevsky district, Tatarstan Republic, Russian Federation
Characteristics * (n=5)			
SiO_2	66.57±8.54	58.54±8.34	12.40±0.71
TiO_2	0.58±0.32	–	–
Al_2O_3	17.04±9.24	6.02±3.27	5.91±1.62
Fe_2O_3	5.51±2.56	1.88±1.04	6.2±1.50
FeO	–	–	–
MnO	0.03±0.02	–	–
CaO	0.80±0.06	14.43±6.24	11.07±12.4
MgO	1.5±0.84	1.86±9.36	–
Na_2O	0.20±0.10	0.20±0.10	–
K_2O	2.57±1.54	1.10±0.62	–
P_2O_5	0.09±0.06	0.08±0.06	0.47±0.25
SO_3	0.42±0.28	–	1.30±0.51
F	–	–	–
CO_2	0.10±0.10	–	–
Loss on ignition (LOI)	5.01±3.21	2.40±1.3	–
Opalkristabolite	–	28.48±10.21	–
Clinoptilolite	–	21.45±9.34	–
Calcite	–	20.14±8.42	–
Montmorillonite	81.00±1.00	23.50±2.14	19.50±2.50
Hydromica	7.00±1.00	–	–
Kaolinite	6.00±0.04	–	9.00±4.35
Quartz	6.00±1.00	6.50±4.80	–
Opaltridymide	–	–	–
Fluorocarbonapatite	–	–	–
Other rocks and inclusions			gibbsite – 5-8, vermiculite – 8-10, saponite – 5-7, amorphous silica – 31-38, halloysite – 2-4, chlorite – 0.5-1, and others – 1-1.5
Cation exchange capacity (meq/100g)	48-51	45-46	17-20
Sorption and moisture capacity, %	58…62	64…67	25…31
Pore structure	Belt-layered, swelling bags	Crystal lattice with internal spaces	Amorphous silica, belt-layered structure, crystal lattice

* All are presented in percentages (%), if not stated otherwise.

3.3 Viability of lactobacilli cells within the liquid synbiotic preparation

We showed that five tested lactobacilli strains do not affect the growth of each other and thus can be successfully used in a multi-strain preparation.

3.2 Characterization of sapropel, bentonite, and zeolite proposed as carrier matrixes in a multi-component preparation

An important attribute of the agrominerals' architecture is the system of regular channels and communicating cavities (pores). Sorption on them is associated with the openwork of their internal structure, which yields a large adsorption volume. Due to the system of channels and cavities that penetrate the crystals of agrominerals, these carrier matrixes have a well-developed internal surface that is accessible to adsorbed molecules but nevertheless usually too small for microbial cells [6, 17]. We examined the distribution of lactobacilli cells within the mineral carrier matrix. For that, we centrifuged the liquid synbiotic preparation at 1000 rpm for 1 minute and then assessed the viability of lactobacilli cells in the upper (liquid) and lower (containing mineral carrier particles) fractions using the drop-plate method. As follows from Table 2, both fractions contained high titers of viable lactobacilli, thus indicating that sapropel, bentonite, and zeolite used as carrier matrixes did not detriment the viability of probiotic lactobacilli. Besides, these data confirm the immobilization of bacteria on the mineral particles of the carrier matrixes.

In liquid probiotics, bacterial cells are in a physiologically active state and are able to colonize the intestine within two hours after administration. Besides, the nutrient base contains valuable bacterial metabolites, such as lactic acid, bacteriocins, secreted proteins, short-chain fatty acids, vitamins, etc. [18]. Thus, liquid probiotics are able to restore the host intestinal microbiota more quickly and efficiently compared to the dry form. Complicated transport and storage requirements are the main commercial disadvantage of liquid preparations since the environmental stresses, especially temperature, pH, and oxygen, can affect the viability of probiotics [19].

Table 2. Viability of lactobacilli in different fractions of the liquid synbiotic preparation obtained by centrifugation for 1 min at 1000 rpm, lg CFU/mL.

Strain	Control*		Sapropel		Bentonite		Zeolite	
	Upper fraction	Lower fraction	Upper fraction	Lower fraction	Upper fraction	Lower fraction	Upper fraction	Lower fraction
FCa3L	9.8	10.0	9.0	10.3	10.0	9.3	7.6	10.0
LS 4-4	9.0	9.0	8.7	10.5	8.7	10.0	8.5	10.5
AG 10	8.1	9.2	8.3	8.2	9.6	9.3	8	9.3
AG16	8.9	8.3	8.2	8.1	7.8	7.4	7.9	8.2
HFD1	8.1	7.7	8.2	8.2	8.1	8.2	7.7	8.2

* For control, 10^9 CFU of lactobacilli were suspended in 5 mL of nutrient base (10% clarified whey, 1% yeast extract, 0.5% sucrose).

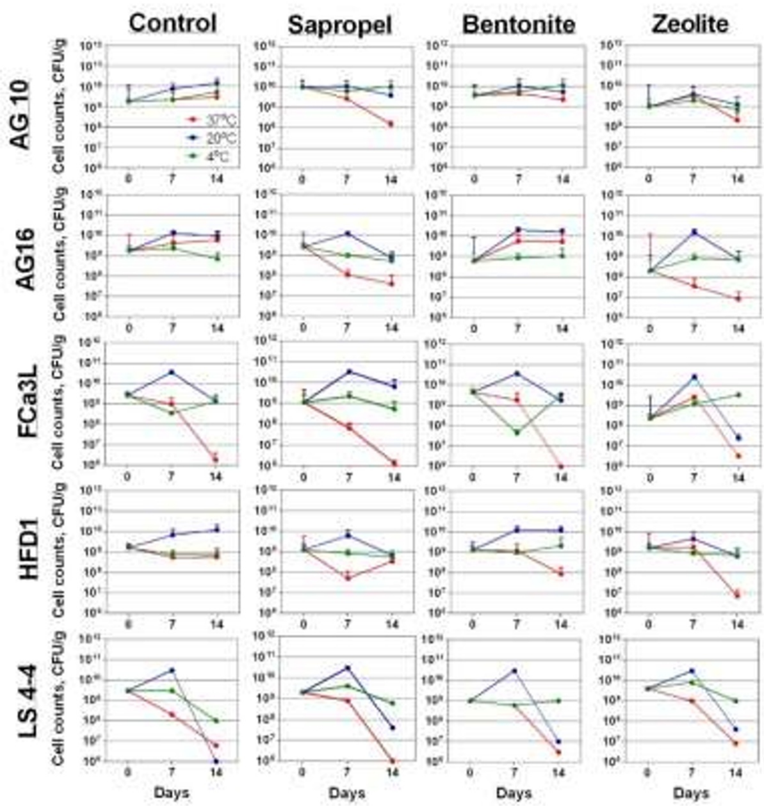

Fig. 1. Viability of *L. plantarum* AG10, *L. rhamnosus* AG16, *L. plantarum* FCa3L, *L. plantarum* LS 4-4, and *L. fermentum* HFD1 in the liquid synbiotics, in which lactobacilli cells are immobilized in sapropel, bentonite, or zeolite. Synbiotic preparations were stored for 14 days at temperature-controlled conditions.

We studied the safety of lactobacilli cells in an experimental series of immobilized probiotics and in a control. The prepared synbiotic preparation was stored at three different temperatures: +4±1 ºC (long-term stability test, UST), 20±1 ºC, or +37±1 ºC (accelerated stability test, HST). The results are presented in Figure 1. We showed that immobilization in sapropel, bentonite, and zeolite did not affect the viability of lactobacilli during two weeks of storage at +4±1 ºC. All five lactobacilli strains selected for inclusion into the liquid synbiotic preparation were preserved in high titers in three mineral carrier matrixes of as much as 10^8-10^9 CFU/g and higher. The viability of *L. plantarum* LS 4-4 in synbiotics was even higher than in the control, indicating the protective effect of immobilization in sapropel, bentonite, and zeolite on the cells. When stored at 20±1 ºC, the viability of lactobacilli was maintained for 7 days, and then the viability of *L. plantarum* FCa3L cells immobilized on zeolite and *L. plantarum* LS 4-4 immobilized on all three carrier matrixes significantly decreased relative to the initial values. The viability of immobilized and control cells of *L. plantarum* AG10, *L. rhamnosus* AG16, and *L. fermentum* HFD1 stayed relatively stable during two weeks of storage at 20±1 ºC. Under the temperature of +37±1 ºC, *L. plantarum* FCa3L and *L. plantarum* LS 4-4 again were the least stable, and their viability significantly (more than two logarithmic orders) decreased in the control and in synbiotics. The viability of *L. rhamnosus* AG16 immobilized on sapropel and zeolite gradually decreased during two weeks. The viability of *L. fermentum* HFD1 immobilized on sapropel on the 7th day and immobilized on zeolite on the 14th day was significantly decreased as compared to the initial state. At +37±1 ºC, only *L. plantarum* AG10 remained stable for 14 days.

High ion-exchange and sorption properties of sapropel, bentonite, and zeolite and suitable bacterial survival during storage are among the main benefits of the developed poultry feed. The obtained data have great potential for practical use in the construction of multi-strain liquid probiotics, in which probiotic lactobacilli are immobilized on mixtures of various mineral matrix carriers.

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