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COMBINING MONTMORILLONITE CLAYS WITH PRO- AND PREBIOTICS TO REVITALIZE THE HUMAN MICROBIAL ECOSYSTEM.

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Abstract: The task of the present paper is to develop scientific bases and technological solutions for creating a combined pharmaceutical composition for normalization of intestinal system function. The assigned task is solved by a combination of energy sorbent of montmorillonite clay with a mixture of probiotic - Lastobacillus plantarum mal and prebiotic inulin. The creation of such a composition is the most effective for normalization of intestinal system function since prebiotic products create a favorable and complete nutrient environment for maintenance and growth of the useful microbial culture of probiotics that were in annibiosis (inactive state), and the enterosorbent of montmorillonite clay provides "adsorption activity" for adsorption of toxic substances. The essence of the technology for obtaining a combined pharmaceutical composition for the normalization of intestinal system function, containing prebiotic and probiotic, as well as enterosorbent - montmorillonitic clay, in which from dry inulin - prebiotic prepare aqueous solution, then it is sterilized by autoclaving, added probiotic culture Lastobacillus plantarum mal, which is restored from the dried state by three times crossing into the MRS - broth, At the same time, Lastobacillus plantarum mal is centrifuged, cells are separated from the cultural liquid and thoroughly mixed before lyophilisation of the substance. The suspension is mixed with a protective medium consisting of milk, sugar and gelatine, the resulting mixture is also subjected to lyophilic drying, the dry amorphous biomass is added to autoclaved montmorrillionite clay and the final product is light brown with a pleasant sour-milk smell. This product serves as a sachet filling station - sachets with additives.

Keywords: entrosorbent, probiotic, prebiotic, bentonite clay, montmorillonite clay, composition, inulin, Lastobacillus plantarum mal, sterilization, centrifugation, sublimation, navbahor meadow.

Relevance of the research: the last three decades have been marked by a marked increase in scientific interest in the microbial ecology of man. More than a century ago, Ilya Mechnikov (Russian scientist, Nobel laureate and professor) postulated that lactic acid bacteria have a beneficial effect on health and promote longevity. He suggested that "intestinal auto intoxication", and the aging it causes, can be reduced by modifying the intestinal microbiota and replacing proteolytic microbes - which produce toxic substances that include phenols, indole and ammonia when digested by beneficial microbes. Mechnikov has developed a diet with the inclusion of milk, fermented bacteria, which he called the "Balgar Bacillus" [1].

Today, the search in rheumatoid clinical trials on humans shows more than 1500 published results on probiotics and almost 350 on prebiotics [1].

Thus, all modern biopreparations are divided into probiotics and prebiotics. Probiotics are preparations containing live cultures of microorganisms capable of stimulating intestinal normoflora. Prebiotics are substances of different non-microbial origin, also capable of stimulating the symbiontic flora of the intestine [2].

Violation of microbial ecology is usually accompanied by contamination of the internal environment with toxic compounds, both exogenous and endogenous.

This is largely due to the loss of normoflora of its full protective function, contributing to the neutralization and elimination of hazardous substances from the body, getting from the external environment and synthesized in the body due to the violation of metabolic processes. Therefore, it is reasonable to include energy sorbents in the treatment systems of patients with dysbiosis [2,10].

At present, there is a huge assortment of enterosorbents of different nature, but not all of them are effective in microbial ecological disorders. Thus, with prolonged use of a number of sorbents, in particular, coal, there may be side effects (constipation, diarrhoea, reduction of vitamin levels of hormones, some microelements, beneficial microorganisms, etc., due to their binding with sorbent), which may involve serious metabolic disruptions. The use of such sorbents, especially activated carbon, is contraindicated in erosive lesions of the mucous membrane of the esophagus, stomach, intestines, as well as gastrointestinal bleeding.

Many sorbents bind bacterial cells, which may lead to the deepening of microecological disorders [10].

The research aimed to develop scientific bases and technological solutions for the creation of combined pharmaceutical composition for normalization of intestinal system function using enterosorbents, probiotics based on local raw material discharges [11-15].

The set goal is achieved by a combination of enterosorbent montmorillonite clink with a mixture of probiotic - Lastobacillus plantarum mal and prebiotic - inulin. The way of creation of such a composition is the most effective for normalization of intestinal system function, since prebiotic products create a favorable and useful nutrient environment for maintenance and growth of useful microbial culture - probiotics, which are in annibaosis (inactive state), and the enterosorbent of montmorillonite clay provides "adsorption activity" for adsorption of toxic substances.

The experimental part.

Materials and methods: as an object of study were used:

1) to obtain inulin the underground part (tubers) of topinambur (Helianthus tuberosus L.), grown in different regions of the Republic of Uzbekistan, inulin concentrate powder of topinambur tubers under the conventional name "Glykoinuvit," purified inulin, was obtained by sedimentation from glykoinuvit;

2) to obtain enterosorbent - montmorillonite clay: alkaline bentonite of Navbakhar deposit in Nawbakhar region of the Republic of Uzbekistan, representing a sabota of light gray

clay with a layered fracture, between the layers there are yellow - brown spots. The pieces of bentonite clays have a waxy luster, greasy to the touch and are easily polished with a nail. Essential samples harden strongly and swell well in water.

3) To obtain probiotic lactobacillus plantarum mal strains are cultivated in MRS environment - broth at 37 oC during 24 hours with constant mixing. Before drying the suspension of lactobacillus strains is mixed with the protective medium in a volume ratio of 1:1.

Protected environment provides increased survival of lactobacterial strains under the influence of toxic for microorganisms biological fluids: gastric juice, bile and juice of the small intestine, allows you to increase the sorption capacity of enterosorbent. Drying of lactobacterial strains is carried out in sublimation units with pre-freezing at -30 oÑ for 16 hours. The obtained masses are hygroscopic amorphous powder of yellow and cream color with a sour smell.

Methods have been used:

-purification;

-centrifugation;

-sterilization;

-IC spectroscopy;

-sublimation drying;

- determination of adsorption capacity;

- determination of the titer of living cells of lactobacterial strains in the composition;

- determination of the total number of bacteria and fungi in the composition.

Devices:

-IR spectra were shot on the device "Avatar-360" (USA);

-Centrifugation was performed on the device K-70;

- sterilization was performed by autoclaving on the device VK-75-01 (Russia).

-liophilic drying was performed on -LZ-9.

Inulin prebiotic was isolated from topinambur tubers (Helianthus tuberosus L.) by the method published by O.Sh. Kadyrov [11]. Topinambur tubers were peeled off, then subjected to cutting and drying and ground in a ball mill. The received powder was dissolved in water, then precipitated with acetane and the received solution was dried in a drying cabinet by hot air at temperature 60-70^{o}C and inulin powder was received.

Purified inulin is a white lightweight powder; unlike starch, it is not coloured with a solution of iodine (it is a fructose polycondensate with a molecular weight of 5000-6000) and does not restore the Felling liquid. Under the action of resobcine in an acidic medium, it is colored red (Selevanov reaction) [11]. In Inulin infrared spectra there are absorption bands 1133,17 sm^{-1}, typical for (C-O-C) bond; 2854,56 sm^{-1}, for $CH_2 - OH$ groups; 934,69 sm^{-1} - for α-polysaccharides; 1032.4 sm^{-1} - for the first time, for HE groups; 3429.26 sm^{-1} - for HE

groups in polymers; and 2924.72 sm^{-1} - for within complex groups. Prebiotic inulin can stimulate symbiotic flora of the intestine.

The probiotic culture Lastobacillus plantarum mal was obtained in the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan, is in the data bank of the Institute's collection. The culture was isolated from the epiphyte microflora of stocky malwa flowers (Malvapusilla) and accepted for storage in the microorganism culture collection on August 29, 2019 (number SKB-343). Probiotic culture of Lastobacillus plantarum mal is restored from the dried state by three times crossing into MRS - broth. Before lyophilization of the substance Lastobacillus plantarum mal is centrifuged at 6000 rpm to separate cells from the cultural liquid.

Live cell titer of lactobacillus strains was determined by serial dilution. For this purpose 3.7 g of 3 kinds of compositions were diluted in 9 ml of physiological solution and thoroughly mixed and titrated by dilution of 1 ml of this suspension in 9 ml of semi-liquid MRS-system.

- MRS composition - broth (g/l):
- Penton 10,0
- Meat extract 10.0
- Yeast extract 5.0
- Twin 80, 1.0 ml
- -sodium acetic acid -5.0
- Ammonium citrate 2.0
- Glucose 20.0
- $MgSO_4$x $7H_2O$ 0.2
- $MgSO_4$x $4H_2$ 0.052
- A-cysteine 0.2
- pH -6.2-6.5
- is sterilized at 1 atm for 15 minutes.
- Manufacturer HiMedia (India)

MRS - semi-liquid medium is prepared by adding 0.075 g agar agar to 100 ml of standard MRS medium - broth. Suspension dilution was performed up to 10^{-9}. After incubation at 37^0C during 24-48 hours, the number of increased suspended lactobacterial colonies was counted.

Montmorillonite clay is included in the technology of pharmaceutical composition, taking into account that it is an enterosorbent and has antidiarrheal activity. Montmorillonite clay (MC) was taken from Navbakhorskoye deposit of RUz and has the following characteristics:

Estimated amount of bentonite clay, which is a lump of silver color, is cleaned from the ground, yellow-brown from mechanical impurities, then roughly cleaned bentonite clay is mixed with 20 liters of treated water, stirred for 1 hour and left to settle the remaining impurities for a day. After that, 4/5 of the upper part of the resulting suspension is separated, leaving the settled part and centrifuged at 6000 rpm. During 30 min. The transparent part of the water from the centrifuge glasses is separated, sandy sediment formed at the bottom of the centrifuge glass is thrown out, and the medium sour creamy layer (~5/4 part) of the suspension is mixed with 12 liters of treated water, stirred into the centrifuge glasses and subjected to subsequent centrifugation at 8000 rev./min. For 45 minutes. Obtaining a homogeneous upper and lower parts of the slurry show a complete cleaning of the clay from fine impurities.

Purified clay enriched with montmorillonite minerals is dried at 413 K in a vacuum drying cabinet with hot air supply for 2 hours. The resulting mass is crushed in a ball mill, sifted, yellowish white powder with a weak earthy smell is obtained. Indicators of the resulting montmorillonite clay:

The pH value of 2% - water suspension: pH 7.0 - 7.6;

Bentonite number: 80 ml;

Colloidality: 90%

Elemental composition, %: Si - 23,5; Al - 7,27; Ca - 0,77; Na - 0,03; Mg - 0,77; P - 0,22; Cl - 0,07; K - 0,04; Ti - 0,17; Fe - 1,29; O - 63,94; Cu - 0,0039; Zn - 0,0036; Y - 0,56; S - 0,27 %.

Radiaactivity: beta sum of radiactivity, Bq/kg - 85; alpha sum of radiactivity Bq/kg - 3,4; isotopic composition Bq/kg – K^{40}- 18,7 (meets the requirements of SampiN № 0093 - 99 (standard 1890 Bq/kg).

Initial experiments on obtaining MC showed that the efficiency of the process is influenced by several factors: the amount of water (hydromolecule) used in the purification process, the frequency of purification, the speed of rotation of the centrifuge. The optimal values of these three factors were found with the help of mathematical planning of the experiment. Hydromodule 1:20, purification ratio 2, centrifuge turnover rate 8000 rpm was established.

The technology of obtaining the pharmaceutical composition is as follows: 5-15% aqueous solutions are prepared from dry inulin, which is sterilized by autoclaving at 0.5 atm for 30 min at 55-60^{o}C. Then the probiotic culture Lastobacillus plantarum mal in a ratio of 1:1 is added, which is restored from the dried state by triple crossing into MRS - broth. Before lyophilisation of the substance, Lastobacillus plantarum mal is centrifuged at 6000 rpm to separate cells from the cultural liquid and mixed thoroughly. This prebiotic and probiotic suspension is then mixed with the protective medium at a ratio of 1:1 by volume with a mixture of prebiotic and probiotic. The protective medium consists of milk, sugar and gelatin. The resulting mixture is subjected to lyophilic drying: cooled to - 60^{o}C, then evaporated at 40 - 60^{o}C under vacuum. Get dry amorphous biomass. After that, add to the biomass sterilized by autoclaving at 121^{o}C for 60 minutes montmorillionite clay in a ratio of 1:3 (1-mixture of prebiotic and probiotic, 3 - montmorillionite clay). They receive the final product - pharmaceutical composition with the conventional name "Prosporbenite", which is an amorphous mass of light brown color with a pleasant sour-milk smell, containing no more than 4% moisture in the composition.

The resulting mass is stirred into a ball mill, cut through a sieve number 5, get a homogeneous powder of gray with a creamy tint, characteristic sour-milky and earthy taste and smell, which is added to the estimated amount of auxiliary substances and packed in sachets, pack 30 sachets in a cardboard box with instructions for use.

The technology for obtaining the pharmaceutical composition is illustrated by the following experiments:

(1) A 15% aqueous solution is prepared from dry inulin, which is sterilized by autoclaving at 0.5 atm at 55-60^{o}C for 30 min. Then probiotic culture Lastobacillus plantarum mal

in a ratio of 1:1 is added, which is restored from the dried state by three times, transferred to MRS - broth. Before lyophilisation of the substance, Lastobacillus plantarum mal is centrifuged at 6000 rpm to separate cells from the cultural liquid and mixed thoroughly. This prebiotic and probiotic suspension is then mixed with the protective medium at a ratio of 1:1 by volume. The protective medium consists of milk, sugar and gelatin. The resulting mixture is subjected to lyophilic drying: cooled to - 60^{o}C, then evaporated at 40 - 60^{o}C under vacuum. Get dry amorphous biomass. After that, add to the biomass sterilized by autoclaving at 121^{o}C for 60 minutes montmorillionite clay in a ratio of 1:3 (1-mixture of prebiotic and probiotic, 3 - montmorillionite clay). They receive the final product - a pharmaceutical composition with the conventional name of preprosporbenite.

(2) The experiment was carried out in the same way as the 1st one, but to obtain a mixture of prebiotic and probiotic 5% aqueous solution of prebiotic inulin was used.

(3) The experiment was carried out in the same way as in 1; however, when the final product was obtained, two parts of component 1 were added to two parts of component 2 at a ratio of 2:1.

(4) The experiment was carried out in the same way as in 1, but one part of component -1 is added to one part of component -2 at a ratio of 1:1.

Then adsorption activity, microbiological and pharmacological properties of the end products were studied and the results obtained were compared (Tables 1 and 2).

Table 1

Sanitary and microbiological parameters of the pharmaceutical composition obtained by the systems 1,2,3 and 4; experiments

№ experiments	Titre strain Lactobacteria, COE/ml	*Entrobacteriaceae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
1	5-7 x 10^{7}	Not found.	Not found.	Not found.
2	5-6 x 10^{5}	Not found.	Not found.	Not found.
3	4-5 x 10^{6}	Not found.	Not found.	Not found.
4	6-7 x 10^{6}	Not found.	Not found.	Not found.

Determination of the total number of bacteria and fungi in compositions. Experiments on determining the total number of bacteria and fungi in compositions were carried out with a two-layer agar method, using media MPA and Saburo as a nutrient medium. For this purpose, each of 3 samples was weighed by 3.7 g and dissolved in 9 ml of physiological solution. After complete dissolution of the compositions, 0.5-1 ml of suspension was introduced into the appropriate medium cooled to 45^{o}C and poured on solid medium in Petri dishes. Cups with seeding are incubated at 30-35^{o}C and 20-25^{o}C (for mushrooms) for 5 days. After 5 days, the number of growing colonies of microorganisms was counted.

Determination and identification of bacteria of Enterobacteriacea family in compositions. To control the compositions for the content of intestinal bacteria by 3.7 g, the compositions were dissolved in 1 ml of sterile water and the entire content was sown with a pipette in 2 cups of Endo media, for the absence of pathogenic and opportunistic bacteria of the intestinal group and blood agar, spilled in a layer of 1.5-2 mm - to identify hemolysis colonies.

On the environment of Endo pathogenic and conditionally pathogenic bacteria of the intestinal group are round, crimson with metallic shine or without it, colonies pink, colorless, shiny, convex in diameter 2-4 mm. At microscopy - not forming sticks.

Determination and identification of bacteria Pseudomonas aeruginosa and Staphylococcus aureus in compositions. To identify synagogue sticks and golden staphylococcus aureus in compositions, 1 dose of preparations was sown in 100 ml of MPB and sown at 30-35^{o}C during 24-48 hours. If there is a growth in the form of turbidity in this medium, we make a loop on agarized media Pseudomonas agar and yolk-salt agar and incubate sowing at 30-35^{o}C for 24-48 hours.

The determination of adsorption activity is presented in Table 2. From the data of the table, we can see that if we place the samples sequentially in ascending adsorption activity, we obtain the following series:

experiment composition - 2 < experiment composition - 4 < experiment composition - 3 < montmorillonite clay < experiment composition - 1. As can be seen, the composition of the experiment - 1 is the most active in terms of adsorption efficiency. We can see that the composition - 1 is 19.5% more active than the montmorillonite itself (154.2 → 184.4 mg/kg). The adsorption activity was reduced by 2,3,4 in the composition of the experiments: 26.98% (154.2 → 112.6 mg/g), 24.90% (154.22-115.8 mg/g) compared to Mg activity. To substantiate the above results from the scientific point of view, let us pay attention to the components of the samples. In particular, composition -1 consists of lily dried montmorillonite clay and a mixture of probiotic and prebiotic biomass, where the greatest amount of inulin (15%-solution). In this composition, the increase in adsorption activity by 19.5% compared to montmorillonite clay can be justified by the inclusion of Lastobacillus plantarum mal and a large amount of fructose (inulin) polycondensate. Lastobacillus plantarum mal is round in shape, usually in width of 0.9-1.2 microns and length of 3-8 microns in the form of a whip; as a result of adsorption on MG due to the increase of active centers increase the specific surface of MG.

Table 2: Adsorption activity results of newly developed propreenterosorbents

Object	Methylene blue mass in the initial solution, mg	Adsorbed methylene blue mass, mg	Adsorbed methylene blue mass, mg/g	Adsorbed methylene blue average mass, mg/g	Metrological description
Control solution	52,5	-	-	-	-
Experimental composition - 1	52,5	18,57	185,7	184,4	S^2=7,752; S= 2,784; ΔX=7,155; ΔX_{cp}=2,921; ε, =3,88%; ε_{cp}=1,58%.
		18,03	180,3		
		18,74	187,4		
		18,18	181,8		
		18,47	184,7		
		18,65	186,5		
Experimental composition - 2	52,5	11,24	112,4	112,6	S^2=1,821; S= 1,349; ΔX=3,468; ΔX_{cp}=1,416; ε, =3,08%; ε_{cp}=1,25%.
		11,05	110,5		
		11,57	115,7		
		11,18	111,8		
		11,62	116,2		
		11,31	113,1		
Experimental composition - 3	52,5	12,52	125,2	125,1	S^2=1,074; S= 1,063; ΔX=2,664; ΔX_{cp}=1,087; ε, =2,12%; ε_{cp}=0,86%.
		12,48	124,8		
		12,67	126,7		
		12,59	125,9		
		12,39	123,9		
		12,43	124,3		
Experimental composition - 4	52,5	11,61	116,1	115,8	S^2=1,577; S= 1,256; ΔX=3,228; ΔX_{cp}=1,317; ε, =2,78%; ε_{cp}=1,13%.
		11,39	113,9		
		11,74	117,4		
		11,59	115,9		
		11,49	114,9		
		11,67	116,7		
MG	52,5	15,46	154,6	154,2	S^2=0,882; S= 0,939; ΔX=2,414; ΔX_{cp}=0,985; ε, =1,56%; ε_{cp}=0,63%.
		15,39	153,9		
		15,54	155,4		
		15,51	155,1		
		15,37	153,7		
		15,29	152,9		

Conclusions:

1. The combination of enterosorbent-montmorillonite clays with a mixture of probiotic Lastobacillus plantarum mal and prebiotic-inulin is the most effective for normalization of intestinal system function that inulin creates a favorable environment for the maintenance and growth of useful microbial culture Lastobacillus plantarum mal, which is in anabiosis (inactive), and montmorillonite clay provides adsorption activity for the adsorption of toxic substances.

2. Enterosorbenite (MG) binds to surface structures of bacterial cells and covers them with a protective layer, protecting them from inhibitory factors: bile acid, pancreatic secrets, bile, enzymes and other harmful compounds.

3. In addition, the montmorillonite clay is characterized by extremely small particles, high hydration during hydration and the ability to form thixotropic highly viscous ashes and gels, which can bind and remove toxins, gases, ions, heavy metals and radionuclides from the body, without affecting the cells of the indogenic microbiota.

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