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SURVIVAL OF MICROORGANISMS FROM MODERN PROBIOTICS IN MODEL CONDITIONS OF THE INTESTINE

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Introduction

In the gastrointestinal tract, exist the protective mechanisms, which prevent overgrowth of pathogenic bacterial and its incorporation. Some of these bactericidal agents are gastric secretions and bile, the integrity of the epithelial and brush border, and immune defense [1].

Three of the main bactericidal agents produced by the gastrointestinal system are pepsin, hydrochloric acid and bile [2]. The acidic environment of the stomach (pH 2-3) and protease (pepsin) destroys the majority of bacteria that enters the stomach with a food. The importance of this acidic environment is evident in studies with patients with hypochlorhydria. Since the bactericidal property of the stomach is weakened in these patients, potentially pathogenic microbes can then migrate to the small intestine and establish itself (growth, vegetate), which is the reason why patients with hypochlorhydria are more prone to infections by *Helicobacter pylori* and *Salmonella spp*. [3].

Bile and pancreatic enzymes are another bactericidal agents that is found in the digestive system, located in duodenum. The microorganisms-probiotics cannot survive during transit through the acidic conditions of the stomach, and resist degradftion by hydrolytic enzymes and bile salts in the small intestine because it's initially not typical for humans. For this reason most of the vegetative forms of probiotics are not available to affect bacterial ecology and metabolism in small intestine. Experimental and clinical studies have shown that the majority of probiotics lose up to 90% of their activity before reaching the small intestine as a result of impact from hydrochloric acid and bile [1].

Lactobacillus, *Bacillus*, *Bifidobacterium*, *Enterococcus* are the most frequently used probiotics in the clinical practices. They have different sensitivity to acidic pH and toxic impact of bile acids [4, 8, 9].

Mostly probiotics contains several microorganisms like lactobacilli with bifidobacteria, sometimes enterobacteria or *Bacillus.* Numerous publications have shown that not all of different combinations of those microorganisms can be stable to impact of the environment of the stomach and intestines [5, 6, 7, 10].

The aim of this study is to compare the ability of microorganisms from 9 probiotic drugs which are available on the pharmaceutical market of Ukraine to survive under model gastric and intestinal environments.

Materials and Methods

9 probiotic drugs used in this study have the following composition:

Lactovit Forte ("Mili Healthcare"). 1 capsule contains $120 \cdot 10^6$ spores of *Bacillus coagulans*, Folic Acid 0,0015 g, Vitamin B1, Vitamin B12, Vitamin B2, Vitamin B3 (Niacinamide), Vitamin B5 (Calcium Pantothenate), Vitamin B6. Serial no. LF171. (Manufacture number). **Linex** ("Sandoz"). 1 capsule contains approximately $1.2 \cdot 10^7$ of lyophilized live lactic bacteria: approximately $4.5 \cdot 10^6$ *Lactobacillus acidophilus* (*sp. L. gasseri*), approximately $3.0 \cdot 10^6$ *Bifidobacterium infantis*, and approximately $4.5 \cdot 10^6$ *Enterococcus faecium*. Serial no. EM3590. **Linex Forte** ("Sandoz"). 1 capsule contains not less than $1 \cdot 10^9$ CFU *Lactobacillus acidophilus (LA-5)*, $1 \cdot 10^9$ CFU *Bifidobacterium animalis subsp. Lactis BB-12*. Serial no. FK5074. **Enterogermina** ("Sanofi-Aventis"). 1 capsule contains: spores of *Bacillus clausii* – $2 \cdot 10^6$. Serial no. M143489. **Enterol** ("Biocodex").1 capsule contains lyophilized *Saccharomyces boulardii* – 250 mg. Serial no.1466. **Hylac Lacto** ("TEVA"). 1 capsule contains lyophilized bacterials like live lacto and bifidobacteria (*Lactobacillus acidophilus, Bifidobacterium lactis and breve, Bacillus coagulans*), also *Streptococcus thermophilus, Lactobacillus bulgaricus* and vitamins of group B. The total number of bacteria at the recommended dose should not be less than $1{,}5 \cdot 10^9$ CFU. Serial no.06/14C. **Probiz** ("Unique Biotech Limited"). 1 capsule contains *Lactobacillus acidophilus* - $2 \cdot 10^6$, *Lactobacillus rhamnosus* – $1{,}5 \cdot 10^6$, *Lactobacillus plantarum* - $1{,}5 \cdot 10^6$, *Lactobacillus reuteri* - $1 \cdot 10^6$, *Lactobacillus casei* - $1 \cdot 10^6$, *Bifidobacterium bifidum* - $1 \cdot 10^6$, *Saccharomyces boulardii* - $2 \cdot 10^6$ – total of $10 \cdot 10^6$ bacterias. Serial no. PZC 008. **Lactiale** ("Farmak"). 1 capsule contains 7 probiotic germs: *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Streptococcus thermophylus*, *Bifidobacterium breve*, *Lactobacillus acidophylus*, *Bifidobacterium longum*, *Lactobacillus bulgaricus* — total of $1{,}0 \cdot 10^8$ in capsule. Serial no.47109. **Yogurt** ("Pharmascience Lab., Inc."). 1 capsule contains $2 \cdot 10^6$ active germs of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*. Serial no.750PF004. For research, probiotic drugs in native form without preliminary cultivation were taken. In such way we simulated the conditions of their entrance to the human body.

Research was conducted in two phases. **Phase 1.** The ability of different strains from 9 probiotic drugs to survive in *in vitro* conditions simulating the passage through the stomach and intestine was evaluated.

The contents of capsules were extracted from all probiotic drugs (or single dose of selected drops) than were placed into 1 ml of distilled water and were inoculated on the agar plates by sectoral method of cultivation. Cell suspensions of each strain were inoculated on sterile agar plates at 37°C for 24 hours. Later, from the different sectors of the plate the number of colonies were determine. Determination of the number of isolated colonies were performed according to standard table [11].

Later initial cell suspensions of each strain were collected by centrifugation (2,000 × g, 10 min) and were incubated at 37°C for 180 min in simulated gastric condition: NaCl – 0,72 g/liter; KCl – 0,05 g/liter; $NaHCO_3$ – 0,37 g/liter; containing 3,0 g/liter pepsin and adjusted at pH 2.5 with HCl. After incubation in simulated gastric conditions, samples were neutralized with NaOH and adjusted at pH 7.0, then cells were collected by centrifugation (2,000 × *g*, 10 min), and washed in sterile distilled water and again aliquots were withdrawn for inoculation on agar plate. The various abilities of the probiotic strains to grow in the presence of concentrated bile salts were also determined. The remaining cell parts from simulated gastric condition were incubated with bile salts (3.0% [wt/vol]), containing 0,1% pancreatin and adjusted to pH 7.0. After cell suspensions were centrifuged (2,000×g, 10 min), washed in sterile distilled water and again aliquots were withdrawn for inoculation on agar plate.

The probiotic drugs used in this study contained different bacterial strains. For this reason, the cultivated strains were grown in different medium. We used MRS agar for inoculation lactobacilli, bifidum agar for bifidobacteria, sabouraud agar for yeasts and molds, endo agar for enterobacteria, blood agar for detection of gram-positive microorganisms (streptococci). All inoculations onto the surface of the agar plates were triplicate.

Phase 2. It was quantitatively assessed the Bacillus coagulans (Lactovit Forte) resistance to simulated gastric and duodenum transit with determination of the number of CFUs by a pour plate technique (the Koch method) [10]. The contents of 1 capsule was diluted in 10 ml of sterile distilled water and serial 10-fold dilutions were made. Than 0.1 ml of suspension was taken from every dilution for inoculation on MRS agar and colony count was performed. From initial cell suspensions 2 ml of sample was taken, centrifuged (2,000 × g, 10 min) and incubated on artificial gastrointestinal conditions. After 3 h of exposure to a simulated gastric juice (pH 2.5.0) and bile solutions were plating serial 10-fold dilutions. 0.1 ml of suspension was taken from every dilution for inoculation on MRS agar and colony count was performed. All inoculations onto the surface of the agar plates were triplicate.

Viable cells were calculated by the formula:

$$N = \frac{C_1 + C_2}{(n_1 + 0{,}1\, n_2) \cdot d} \text{ CFU}$$

Where:

$C_1 + C_2$ – the sum of counted colonies on the plates of two adjacent dilutions;

n_1 – the quantity of plates from first dilution;

n_2 - the quantity of plates from second dilution;

d – the coefficient for first dilution;

0,1 – the coefficient, considering the first and second the multiplicity of dilution.

Statistical analysis. Statistical analysis comprised significant testing of the difference between means using a two tailed Student's t-test at the level 0.05.

Results and discussion

The effect of simulated gastric and duodenal transit on the viability of selected species from different probiotic drugs is presented in Table 1.

All studied probiotics were grown on appropriate media. The probiotics Enterol and Probiz, which contain *Saccharomyces boulardii* were grown on sabouraud agar, Linex and Linex Forte after inoculation on MRS agar for lactic bacteria, and Linex Forte after inoculation on bifido agar and engo agar were grown for high yield (10^{7-8} CFU). Its show that those probiotic drugs really contain high amount of viable cells. Probiotic Yogurt on MRS agar (10^{6-7} CFU), *Bacillus coagulans* - the drug component of Lactovit Forte (10^{5-6} CFU), Lactiale on MRS agar (10^{5-6} CFU) showed somewhat lower viability. The viability of Hylac Lacto strains was the least (10^{4-5} CFU). During inoculation of Hylac Lacto, they were grown only on MRS agar. The growth on bifido agar and blood agar (for detection of streptococci) was not observed.

After incubation in simulated gastric juice *Lactobacillus* and *Bifidobacterium* did not survive. Also the growth was absent after inoculation of such probiotics as Linex on MRS, bifido and endo agar, Linex Forte on MRS and bifido agar, and Lactiale and Yogurt on MRS agar and blood agar.

A monostrain probiotics Enterol and Lactovit Forte have shown full resistance in simulated gastric juice. The growth performance of *Saccharomyces boulardii* as component of probiotic Enterol on sabouraud agar was on the same level (10^{7-8} CFU) as was before exposed to the simulated gastric juice. The number of germinated spores of *Bacillus coagulans*: a component of Lactovit Forte after cultivation on MRS agar was not reduced during exposure to pepsin and hydrochloric acid.

A strains of probiotic drug Probiz had a high level of surviving in the gastric juice environment. The growth was determined on all three medium - sabouraud, MRS and bifido agar. After exposure to artificial gastric juice, it was noticed non significant decrease in viable numbers of bifidobacteria from 10^{7-8} till 10^{6-7} CFU.

Table 1. The effect of simulated gastric and duodenal transit on the viability of probiotics (n = 3)

Probiotic drugs	Microorganisms	CFU in 1 ml of suspension		
		Initial	After exposure of gastric juice	After exposure of bile
Lactovit Forte	*Bacillus coagulans*	L – (2,0±1,5) $\cdot 10^6$	L – (4,0±3,0) $\cdot 10^5$	L – (5,3±2,6) $\cdot 10^5$
Linex Forte	*Lactobacillus acidophilus* *Bifidobact. animalis subsp. Lactis*	L – (3,8±3,1) $\cdot 10^7$ B – (5,3±2,6) $\cdot 10^5$	L – 0 B – 0	L – 0 B – 0
Linex	*Lactobacillus acidophilus* *Bifidobacterium infantis* *Enterococcus faecium*	L – (5,2±2,7) $\cdot 10^5$ B – (2,0±1,5) $\cdot 10^7$ E – (5,3±2,6) $\cdot 10^6$	L – 0 B – 0 E – 0	L – 0 B – 0 E – 0
Enterogermina	*Bacillus clausii*	L – (3,7±3,2)$\cdot 10^2$ B – (7,0±3,0)$\cdot 10^6$	L - 0 B – (2,3±1,3) $\cdot 10^5$	L - 0 B – (8,3±1,7) $\cdot 10^4$
Enterol	*Saccharomyces boulardii*	S - 4,0$\cdot 10^7$	S - 5,3$\cdot 10^7$	S - 2,3$\cdot 10^7$
Hylac Lacto	*Lactobacillus acidophilus* *Bifidobacterium lactis* *Bifidobacterium breve* *Bacillus coagulans* *Streptococcus thermophilus* *Lactobacillus bulgaricus*	L – (5,3±2,6) $\cdot 10^4$ B – 0 Bl – 0	Л – (2,0±1,5) $\cdot 10^5$ B – 0 Bl – 0	Л – (6,7±1,7) $\cdot 10^4$ B – 0 Bl – 0
Probiz	*Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Lactobacterium plantarum* *Lactobacillus reuteri* *Lactobacillus casei* *Bifidobacterium bifidum* *Saccharomyces boulardii*	L – (5,3±2,6) $\cdot 10^7$ B – (2,3±1,3) $\cdot 10^7$ S – (3,7±3,3) $\cdot 10^7$	L – (7,0±3,0) $\cdot 10^6$ B – (2,0±1,5) $\cdot 10^6$ S – (7,0±3,0) $\cdot 10^5$	L – (5,3±2,6) $\cdot 10^5$ B – (7,0±3,0) $\cdot 10^5$ S – (2,3±1,3) $\cdot 10^5$
Lactiale	*Lactobacillus casei* *Lactobacillus rhamnosus* *Streptococcus thermophilus* *Bifidobacterium breve* *Lactobacillus acidophilus* *Bifidobacterium longum* *Lactobacillus bulgaricus*	L – (4,0±3,0) $\cdot 10^5$ Bl – (6,7±1,7) $\cdot 10^4$	L – 0 Bl – 0	L – 0 Bl – 0
Yogurt	*Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Streptococcus thermophilus* *Lactobacillus bulgaricus*	L – (5,3±2,6) $\cdot 10^6$ Bl – (7,0±3,0) $\cdot 10^3$	L – 0 Bl – 0	L – 0 Bl – 0

L – MRS-agar for lactobacilli; B – bifido agar for bifidobacteria; S – sabouraud agar to isolate yeasts and molds; E – endo agar for isolation enterobacteria; Bl – blood agar for detection of gram-positive microorganisms

Probiotic Hylac Lacto showed different results on different agar. The number of viable cells after incubation in artificial gastric juice and cultivation on MRS agar declined in population and contained $10^{4\text{-}5}$ CFU. But on Bifido and Blood agar strains did not remain viable after passing the gastric contence.

In the same time the viability of strains which contained probiotic drug Enterogermina were significantly lower. The survival of spore-forming probiotic *Bacillus clausii* after passing the gastric compartment was reduced from 10^7 CFU till 10^5 CFU.

The effect of bile was investigated on both the growth and survival of bifidobacteria that were able to survive well in the gastric model. To simulate the sequence of environmental stresses the bacteria would face in gastrointestinal tract, bacteria were first treated in the *in*

vitro gastric model described previously, followed immediately by exposure to a physiological concentration of bile. The strains of Linex, Linex Forte, Lactiale and Yogurt after exposure to bile were not survived. The growth of *Bacillus clausii* (Enterogermina) was reduced till 10^{4-5} CFU. The number of viable cells of Probiz drug passed both exposure were decrease till 10^2 CFU under cultivation on sabouraud agar. The same situation with probiotic drug Probiz, quantity of viable cells was reduced till 10^2 CFU under cultivation on MRS and Bifidum agar. Grown cells microscopy identified *Saccharomyces boulardii*. Any of other strains did not survive after exposure.

The number of bacteria in Enterol, Linex Forte and Hylac Lacto did not change after cultivation on agar as a result of exposure in the gastric model. It showed that just *Saccharomyces boulardii* from Enterol can survive. The same result was shown by *Bacillus coagulans* from Lactovit Forte. Hylac Lacto a multistrain probiotic grew just on MRS agar in the same measure after passing gastric juice and exposure of bile and growth was absent on bifido and blood agar. So the surviving bacteria was spore-forming *Bacillus coagulans*. The identity of *Bacillus coagulans* was determined by microscopic detection of grew colonies after incubation with the bile.

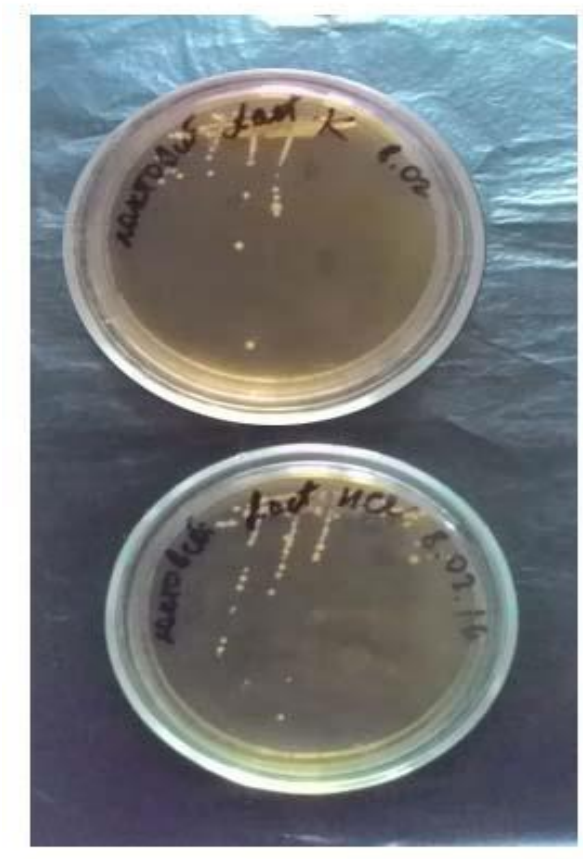

Fig. 1. Lactovit Forte, MRS agar. The control, after impact of simulated environment of the stomach and duodenum

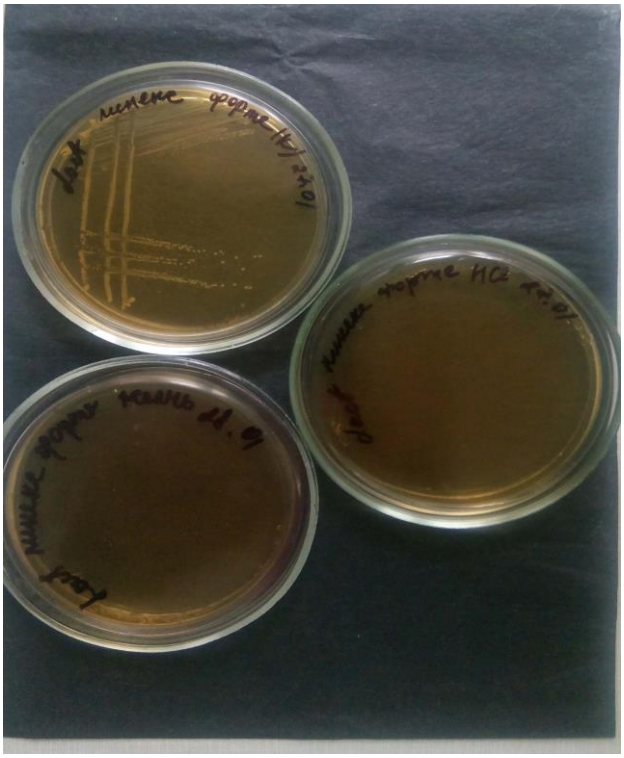

Fig. 2. Linex Forte, MRS agar. The control, after impact of simulated environment of the stomach and duodenum

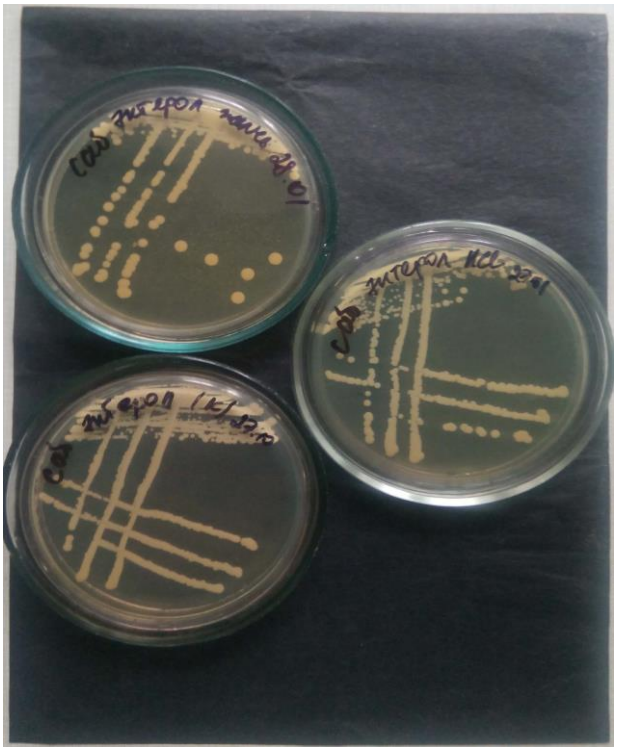

Fig. 3. Enterol, Saburo agar. The control, after impact of simulated environment of the stomach and duodenum

Thus probiotic drugs containing *Bacillus coagulans* and *Saccharomyces boulardii* were stable to impact of gastric juice and bile acids, *Bacillus clausii* was partially resistant.

On the basis of these data we can make the conclusion that probiotic drugs containing lactobacilli and bifidobacteria cannot survive under exposure of bactericidal agents produced by the gastrointestinal system as pepsin, hydrochloric acid and bile. Probiotic drug Enterol which contains *Saccharomyces boulardii* and Lactovit Forte which contains spore-forming *Bacillus coagulans* survive in full measure. In the same time in a multistrain probiotics Probiz and Hylac Lacto (lactobacillus, bifidobacterium and enterobacteria) there were determined only *Bacillus coagulans* and *Saccharomyces boulardii* after passing artificial environment.

Resistance of *Bacillus coagulans* spore to impact of hydrochloric acid and bile was proved by colony count after serial dilutions and inoculation on agar.

After incubation the contents of Lactovit Forte capsule on MRS agar, the growth of colonies were detectable for 2 days. According to literature in the conditions of the body the spore of *Bacillus coagulans* start to grow faster after being exposed to the natural activation. *Bacillus coagulans* cell morphology was determined by microscopy. It was typical, the colonies were small, round, convex, milky, smooth, and with even edges.

In the result of first and second *Bacillus coagulans* dilution inoculation on Petri plates, there was abundant growth of *Bacillus coagulans* and counting of colonies was not possible. In the third dilution (10^{-3}) separate colonies which were densely located were determined. At this dilution the number of colonies on the plates were 286,7±20,3 (Table 2). With further dilutions, the number of colonies decreased. CFU was counted based on results of 4 and 5 dilutions and was $9,27 \cdot 10^6$ cells. Therefore just 1/10 part was incorporated in drug spores were sprouted on MRS agar under these culture conditions.

Table 2. The effect of simulated gastric and duodenum transit on *Bacillus coagulans* (n = 3)

Dilutions	The number of colonies		
	Initial	HCl + pepsin	Bile + pancreatin
10^{-1}	AG	AG	AG
10^{-2}	AG	AG	AG
10^{-3}	286,7±20,3	243,3±28,5	275,3±22,5
10^{-4}	91,7±11,6	81,3±11,6	86,0±8,2
10^{-5}	10,3±2,8	8,3±2,6	10,3±2,0
10^{-6}	1,7±0,9	1,0±0,6	2,7±0,3
CFU	$9,27 \times 10^6$	$8,15 \times 10^6$	$8,76 \times 10^6$

*AG – abundant growth; * - the reliability of the difference in relation to the initial indicators; ** - the reliability of the difference in relation to HCl + pepsin indicators*

After exposure in artificial gastric juice the number of CFU significantly did not change and was $8,15 \cdot 10^6$. It was the same result like a previous exposure. The same date was obtained after exposure in bile, it was $8,76 \cdot 10^6$ CFU. This indicates that spore of *Bacillus coagulans* is totally resistant to impact of aggressive factors of gastric and duodenum. Passing further into the intestine the spores start to grow and can exert probiotic effects there.

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SURVIVAL OF MICROORGANISMS FROM MODERN PROBIOTICS IN MODEL CONDITIONS OF THE INTESTINE

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Introduction. The staye of intestinal microflora affects the work of the whole organism. When composition of normal ibtestine microflora changes, its restoration is required. In our days a wide variety of probiotic drugs are available on the market which can be used to solve this problem. Most bacteria having probiotic properties represent the families *Lactobacillus* and *Bifidobacterium*, which have poor resistance to acidic content of the stomach and toxic effects of bile salts. Various studies have clearly shown that in a person with normal acidic and bile secretion, the lactobacilli and bifidobacteria are not detected after the passage through the duodenum, i.e., they perish before reaching the small intestines. In this study we compared the survival of different microorganisms which are contained in 9 probiotic drugs in a model of gastric and intestinal environments. **Material and methods.** In the laboratory of SI: "Mechnikov Institute Microbiology and Immunology, National Ukrainian Academy Medical Sciences" the *in vitro* experiments have been evaluated to test the ability of different probiotic bacteria which were contained in 9 probiotic drugs to survive the impact of the model environment of the stomach and duodenum. *Bacillus coagulans* persistence was evaluated under impact of simulated environment of the stomach and duodenum, it also was assessed by the quantity of CFU by incubation on culture medium. The following were studied: *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus reuteri, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus bulgaricus, Bifidobacterium bifidum, Bifidobacterium longum , Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium animalis subsp. Lactis BB-12, Saccharomyces boulardii, Bacillus coagulans, Bacillus clausii, Enterococcus faecium.* Microorganisms were incubated for 3 hours in a model environment of the stomach (pepsin 3 g / l, hydrochloric acid of 160 mmol / l, pH 2.3), later after centrifugation and washing, they were incubated for 3 hours in intestinal model environment (bile salts 3% pancreatin 0.1%, pH 7.0). Inoculation was performed before incubation, after incubation in the gastric medium and after incubation in intestinal medium. We used the medium corresponding to the studied genus of bacteria - MRS-environment for lactobacilli, bifidum for *Bifidobacterium*, sabouraud medium for the isolation of yeasts and fungi and endo agar for the isolation of *Enterobacteriaceae*. We assessed the quantity of CFU before and after impact. **Results and discussion.** After incubation in a simulated gastric environment, bacteria of the type *Lactobacillus* and *Bifidobacterium* did not survive and were not defined. Only *Bacillus coagulans* and *Saccharomyces boulardii* were resistant. These microorganisms grew after incubation in the same amount as before incubation - 10^{5-6} and 10^{7-8} CFU respectively. *Bacillus clausii* also survived in these conditions, but to a lesser extent: initially - 10^7 CFU, after incubation - 10^5 CFU. After staying in model environment of the duodenum *Bacillus coagulans* and *Saccharomyces boulardii* were still fully viable, and the number of germinating *Bacillus clausii* bacteria decreased by an order - up to 10^4 CFU. **Conclusion.** The probiotics containing *Bacillus coagulans* and *Saccharomyces boulardii* showed complete resistance to the impact of the model environment of the stomach and duodenum, *Bacillus clausii* was partially resistant. It leads to conclusion that probiotic drugs containing lactobacilli and bifidobacteria, cannot withstand the aggressive environmental influence of the stomach and duodenum and become inactivated under their influence. Probiotic drugs Enterol containing yeast *Saccharomyces boulardii*, and Laktovit Forte containing the spore-forming bacterium *Bacillus coagulans* are completely resistant to the action of the model environment of the stomach and duodenum.
Keywords: probiotics, intestine, stability to acids and bile.