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Results of the microbiota assessment in experimental ulcerative colitis

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ABSTRACT

Background. The increased incidence of inflammatory bowel disease (IBD) in the world and the lack of consensus on the causes and development mechanisms of IBD are the key factors that determine the relevance of the study. According to some authors, in the pathogenesis of the development and occurrence of ulcerative colitis, one of the leading causes is a change in the composition of the colon microflora and the impact of products of microbial metabolism on the enteric nervous system and intestinal motility.

The aim was to study the qualitative and quantitative changes in the colon microbiota in rats when modeling ulcerative lesions.

Materials and methods. The experimental study was carried out using male Wistar rats (n = 24). An original model of ulcerative colitis was used.The quantitative and qualitative composition of the parietal microflora of the distal colon was determined.

Results. In the ulcerative colitis model, changes in the qualitative and quantitative composition of the parietal microflora of the colon were revealed. On the 3rd day, there was a decrease in *Lactobacillus* ssp. and *Escherichia coli*, as well as growth of fungal microflora and appearance of opportunistic bacteria. The changes were progressive in nature, and by the 7th day of the study, marked reduction of the total parietal concentration of the normal flora bacteria and an increased percentage and absolute number of opportunistic microorganisms. By the 10th day of the experiment, with a small increase in the total number of parietal bacteria, the predominant microorganisms were *Bacteroides* ssp. (26.8%) and *Peptococcus* ssp. (27.6%).

Key words: ulcerative colitis, bacteriology, microflora, experiment.

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Результаты оценки микробиоты в условиях экспериментального язвенного поражения толстой кишки

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РЕЗЮМЕ

Введение. Язвенный колит – хроническое рецидивирующее системное воспалительное заболевание с преимущественным поражением слизистой оболочки толстой кишки. Каждый год регистрируется до 20 новых случаев заболевания на 100 тыс. населения, в основном среди лиц трудоспособного возраста. По мнению ряда авторов, в патогенезе развития и возникновения заболевания одну из ведущих причин играет изменение в составе микрофлоры толстой кишки, а также продукты их метаболизма, воздействующие на энтериновую систему и моторику кишечника.

Цель. Изучить показатели микробиоты толстой кишки у самцов крыс линии Вистар при моделировании язвенного поражения.

Материалы и методы. Экспериментальное исследование выполнено с использованием самцов крыс линии Вистар (n = 24). Предложен оригинальный способ модели язвенного колита. Определен количественный и качественный состав пристеночной микрофлоры дистального отдела толстой кишки.

Результаты. Выявлены изменения качественного и количественного состава пристеночной микрофлоры толстой кишки: на 3-и сут отмечали снижение концентрации *Lactobacillus* ssp. и *Escherichia coli*, а также рост грибковой микрофлоры, появление представителей условно-патогенной микрофлоры. Изменения носили прогрессирующий характер, и уже к 7-м сут выявляли выраженное снижение общей пристеночной концентрации бактерий нормофлоры и повышение процентного и абсолютного числа представителей условно-патогенной микрофлоры. К 10-м сут эксперимента при малом увеличении общей численности пристеночных бактерий преобладающей микрофлорой являются *Bacteroides* ssp. (26,8%) и *Peptococcus* ssp. (27,6%).

Ключевые слова: язвенный колит, бактериология, микрофлора, эксперимент.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования.

Соответствие принципам этики. Исследование одобрено комитетом по биомедицинской этике ИНЦХТ (протокол № 7 от 26.06.2017).

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INTRODUCTION

The incidence rate of inflammatory bowel dis- ease (IBD) in the world and the absence of a consensus concerning the reasons and mechanisms of the IBD development are the key factors determining the study relevance. One of the pathogenetic mechanisms of the IBD development is considered to be the abnormality in the intestine microflora composition [1]. In normal conditions, the microflora of the colon contains over 500 species of different microorganisms, 92–95% of which is the so-called obligate microflora represented by *Bifidobacterium, Lactobacillus*, and nonpathogenic *Escherichia coli*

(*E. coli*). Normal microflora executes such functions as regulation of water and salt metabolism, gas composition, metabolism of all nutrients, colonization resistance, detoxication of exogenetic and endogenic substrates, morphokinetic action, mutagenic and immunogenic functions, and energy production for host cells, and, therefore, is an essential "organ" of our body [2–4].

It is known that disruption of the normal intestinal microbiota balance results in occurrence of pathologic processes in mucous membranes. Quantitative and qualitative composition of the colon microbiota depends on a set of both endo- and exogenous factors. Thus, quantitative and qualitative composition of microflora, even for representatives of one species, can vary greatly, within wide limits, which is determined both by the place of residence and diet. Microflora of all patients with IBD is characterized by development of dysbacteriosis, in which a decrease in the normally prevailing *Firmicutes* and *Bacteroidetes* types and an increase in the *Proteobacteria* E21 type are observed. However, no uniform changes in the composition of intestinal microflora characterizing the specific features of this pathology were detected [1].

Changes in the composition of intestinal microflora in patients with irritable bowel syndrome mainly consist in reduction of *Lactobacilli* and *Bifidobacteria* and prevalence of aerobic organisms over anaerobic ones.. Moreover, the parietal composition of microflora in patients with irritable bowel syndrome includes a greater variety of microorganisms than in healthy people [5, 6]. During the development of dysbacteriosis caused by a certain etiological agent, a specific change is the detection of the etiological agent in both the luminal and parietal microflora.

The aim of the work was to study the qualitative and quantitative changes in the composition of the parietal microflora of the colon under conditions of the ulcerative colitis modeling.

MATERIALS AND METHODS

The experimental study was performed at the vivarium of Irkutsk Scientific Center of Surgery and Traumatology (vivarium of the I category, Veterinary Certificate 238 No. 0000023 of 28.11.2015, veterinary service of Irkutsk Region) on male Wistar rats with the weight of 280–350 g (n = 24) and aged over 6 months. Among the experimental animals, 6 rats made up a control group of healthy animals required to obtain reference values. In the group of rats with modeled ulcerative colitis, 6 animals were bred for each microbiota examination term. The animals were kept in the vivarium with free access to water and food in accordance with the national standard "Keeping Experimental Animals in Nurseries of Research Institutes".

The experiments were performed according to the standards of humane animal care, which are regulated by the "Rules of Work Performance with Use of Experimental Animals" (Appendix to the Order of the Ministry of Health of the USSR No. 755 of 12.08.1977 and No. 48 of 23.01.1985 "On Control over Work Performance with use of Experimental Animals", provisions of the World Medical Association's Declaration of Helsinki adopted in 1964 and amended in 1975, 1983, and 1989) according to the Protocol approved by the Bioethics Committee. All surgical interventions were performed under aseptic conditions and general anesthesia.

An original method for modeling ulcerative lesion of rat colonic wall was developed [7]. The parietal microflora of colon biopsy material was assessed by bacteriological methods including the study of qualitative and quantitative composition of aerobic, facultative anaerobic, anaerobic microflora, fungi of the genus *Candida* within certain time intervals of the experiment using highly selective solid and liquid media, the aerobic and anaerobic cultivation techniques, diagnostic short-term tests and the semi-automatic microbiological analyzer "ATB Expression" (Biomerieux, France) in accordance with the current regulatory documentation (OST 91500.11.0004–2003. Patient Management Protocol. Intestinal Dysbacteriosis. Order of the Ministry of Health of the Russian Federation No. 231 of 09.06.2003). The material was collected according to the methodological guidelines MU 4.2.2039–05 "Technique for Biomaterial Collection and Transportation in Microbiological Laboratories". The samples were delivered to the laboratory within an hour. Upon weight determination (with accuracy to 0.001 g), the biopsy material was thoroughly homogenized in 0.85% sodium chloride solution at the rate of 1:10. Ten-fold dilutions of the homogenate up to 10^{-8}–10^{-11}(weight / volume) were prepared from the produced suspension.

Cultures for an extended set of media for aerobic and facultative anaerobic bacteria (Endo's, Ploskirev's, Levin's media, egg yolk salt agar, meat infusion agar with 5% blood, Bismuth sulphite agar, etc.) were performed from thecorresponding dilutions. For anaerobe recovery, fluid thioglycollate medium, Blaurock medium, Wilson – Blair medium, and anaerobic blood agar were used, to which base 5% laked blood, 10 μg / ml vitamin K and 5 μg / ml hemin were added. For fungus recovery, Sabouraud's medium was used. Anaerobic microflora was cultivated under strict anaerobic conditions using the GEN-box anaer devices (BioMerieux, France) and the gas-generating kit (HiMedia Laboratories, India).

The results were recorded in corresponding time intervals. The result was expressed in CFU / g considering the initial weight of the biopsy material and the homogenate dilution ratio.

The obtained data were presented in the form of median percent with lower and upper the median *Me* with lower and upper quartiles (Q_1–Q_3). The significance of the differences in the obtained data ($p \leq 0.05$) in the compared samples were determined by the Mann – Whitney U test. Statistical processing of the results was performed with the Statistica 10.0 for Windows software package (License No. AXAR402G263414FA-V) [8].

RESULTS AND DISCUSSION

The qualitative and quantitative composition of parietal microflora on the 3rd, 7th, and 10th days of the experiment was assessed using the developed original experimental model of ulcerative lesion of the distal colon segment with wall ischemia and maintained inflammation. Modeling was performed by surgical intervention with parietal recovery, legation and transection of the direct artery branches of the colon with accompanying veins within 3 cm from the urinary bladder base for ischemic damage of the distal segment of the colonic wall. One day after the intervention, the experimental animals received 1% aqueous solution of dextran sulfate sodium orally by free drinking throughout the whole experiment to maintain inflammatory process in the colonic wall.

When assessing quantitative and qualitative composition of the colon parietal microflora of healthy animals, it was determined that 95% of the parietal microbiota was obligate flora represented by *Lactobacillus* spp. (48.7 %), *Bacteroides* ssp. (31.7%), *Peptococcus* spp. (14.6%), *Enterococcus faecium* (2.9%), gram-positive rods (1.2%), *Bifidobacterium* spp. (0.7%), *E. coli* (0.1%), and others (0.1%) (Fig.1, Table 1).

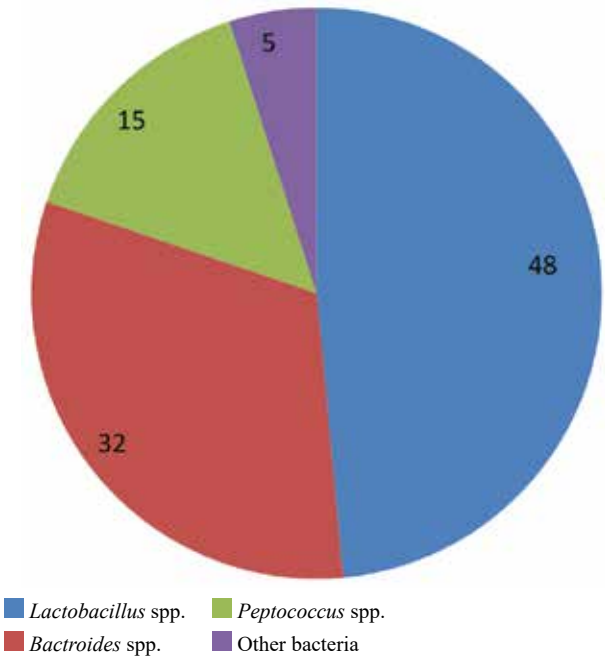

Fig. 1. The percentage of the parietal microflora of the colon in healthy Wistar rats

According to the results of the bacteriological examination, by the 3rd day of the experiment, it was determined that against induced ulcerative colitis, reduction of the total bacterial count was noted by more than 2 times in comparison to healthy animals. In absolute numbers, it was reflected in reduction of the quantity from 8.21×10^9 to 3.92×10^9 due to a significant decrease in the number of *E. coli* (10^6–10^4), *Enterococcus faecalis* (10^7–10^4), gram-positive rods (10^5–10^0), and *Bacteroides* ssp. (10^8–10^5), with an accompanying significant increase in *Bifidobacterium* spp. (10^7–10^8) ($p \leq 0.05$). The comparative analysis of the percentage of parietal microflora in the animals with induced ulcerative colitis on the 3rd day of the experiment showed an increase in *Bifidobacterium* spp. from 0.7% to 38.3 %, *Lactobacillus* spp. from 48.7% to 61.3 %, as well as a reduction of facultative microflora representatives from 51.3 % to 0.4% in comparison with healthy animals.

Table 1

The quantitative and qualitative composition of the colon parietal microflora in the original model of ulcerative colitis (Lg CFU / g), *Me* (Q_1–Q_3)

Parameter		Norm	3rd day	7th day	10th day
		Lg CFU / g			
		Frequency, %			
	1	6.1 (6.0–6.3)	3.7 (3.0–4.3)*	1.7 (0–4.0)*	3.8 (3.6–3.9)*
		100%	100%	50%	100%
	2	3.0 (0–5.0)	0 (0–4.0)	0 (0–0)	0 (0–0)
		66.7%	33.3%		
	3	3.5 (3.5–4.0)	3.0 (0–4.0)	0 (0–0)*	0 (0–0)*
		100%	66.7%		
	4	7.0 (5.0–7.2)	3.75 (3.5–3.9)*	0 (0–0)*	0 (0–0)*
		100%	100%		
	5	1.3 (0–3.0)	1.5 (0–3.6)	0 (0–3.0)	0 (0–0)
		50%	50%	33.3%	
	6	5.0 (5.0–6.3)	0 (0–4.0)*	0 (0–0)*	0 (0–5.0)
		100%	33.3%		33.3%
	7	0 (0–5)	3.0 (0–3.2)	0 (0–0)	0 (0–0)
		33.3%	66.7%		
	8	7.0 (7.0–7.0)	8.0 (8.0–8.0)*	3.0 (0–6.0)*	5.5 (5.0–6.0)*
		100%	100%	50%	100%
	9	9.0 (5.0–9.0)	8.0 (8.0–9.0)	2.5 (0–5.0)*	6.0 (5.0–6.0)
		100%	100%	50%	100%
	10	8.0 (8.0–8.3)	5.0 (5.0–6.0)*	2.5 (0–5.5)*	6.0 (5.0–6.3)*
		100%	100%	50%	100%
	11	0 (0–0)	0 (0–6.0)	0 (0–0)	0 (0–0)
			33.3%		
	12	0 (0–0)	0 (0–0)	0 (0–0)	2.5 (0–5.3)
				16.6%	50%
	13	0 (0–8.3)	0 (0–6.0)	0 (0–0)	6.0 (5.6–6.0)
		33.3%	33.3%		100%

Note: 1 – E. *coli*; 2 – *Proteus mirabilis*; 3 – *Citrobacter freundii*; 4 – *Enterococcus faecalis*; 5 – *Staphylococcus epidermidis*; 6 – *gram-positive rods;* 7 – *Candida* spp.; 8 – *Bifidobacterium*; 9 – *Lactobacillus;* 10 – *Bacteroides* ssp.; 11 – *Actinomyces* spp.; 12 –*Vellonella* spp.; 13 – *Peptococcus* spp*;* * – statistically significant difference by the Mann – Whitney test in comparison with normal values ($p_U \leq 0.05$).

It is known that *Lactobacillus* can produce lactic acid, hydrogen peroxide, lysozyme, and substances with antibiotic activity, such as reuterin, plantaricin, lactocidin, and lactoline. Along with *Bifidobacteria*, *Lactobacilli* interact with enterocytes and stimulate body defense mechanisms, increasing the regeneration rate of mucous membrane, having an effect on synthesis of antibodies to related but pathogenic microorganisms, and activating phagocytosis and synthesis of lysozymes, interferons, and cytokines. *Lactobacilli* produce a numbe*r* of hydrolytic enzymes, particularly, lactase and decomposable lactose, thus preventing the development of lactase deficiency. Moreover, *Lactobacilli* maintain pH of the colon at 5.5–5.6 [9, 10].

When inducing ulcerative colitis during the experiment, in was revealed that on the 3rd day, the lactose negative bacteria appeared accounting for 34.8% from total number of *E. coli*, which is an initial evidence of developing dysbiosis. The significant reduction of the colon bacilli also indicates the developing dysbiosis. It is known that *E. coli* benefits the host organism by synthesizing vitamin K and preventing the development of pathogenic microorganisms in the intestine [11]. A drastic increase of *Bifidobacteria* indicates development of

the compensatory reaction directed to maintain the normal condition of parietal microflora and protective properties of the intestinal wall. *Bifidobacteria* provide physiological defense of the intestinal barrier from the ingress of microbes and toxins into the internal environment and have high antagonist activity in relation to the pathogenic microorganisms and opportunistic pathogens due to generation of organic fatty acids.

In 33% of the examinations, the occurrence of *Actinomyces* was detected. Insertion of *Actinomyces* into the colon mucosa can result in generation of the infectious granuloma reaching surrounding tissues and forming apostasis and fistulae. *Staphylococcus* also contributes to the formation of the suppurative process. An increase in its total concentration of which almost by 3.5 times was noted by the 3rd day of the experiment. *Actinomyces* antigens result in a specific sensibilization and allergic alteration of the body (hypersensibilization of delayed or tuberculin type), as well as antibody formation (complement-fixing, agglutinins, precipitins, etc.) (Fig. 2).

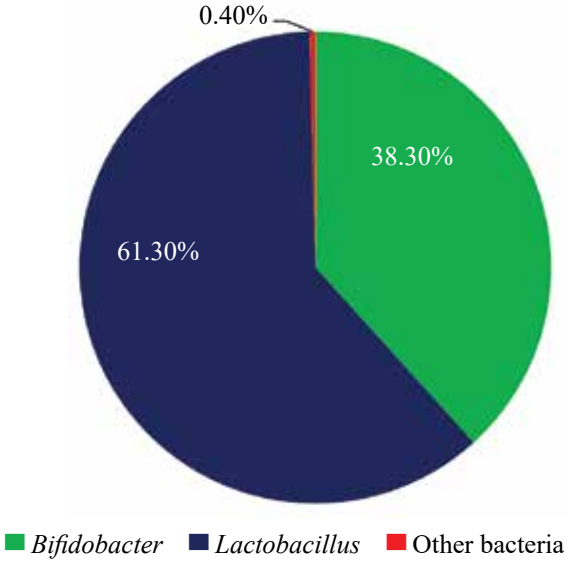

Fig. 2. The percentage of the parietal microflora of the colon in rats under conditions of modeled ulcerative colitis on the 3rd day

By the 7th day of the experiment, against the background of ischemia and reduction of protective properties of the intestinal wall, a significant decrease in the absolute total number of bacteria to 6.25×10^6 was identified in comparison with the indices of healthy animals (Fig. 3). Moreover, a significant difference with the results of the 3rd day of the experiment ($p = 0.025$) was determined. In comparison with indices of microbiota for healthy animals, a significant decrease in the concentrations of *E. coli* (10^6–10^2), *Bifidobacterium* spp. (10^7–10^3), *Lactobacillus* spp. (10^9–10^3), *Bacteroides* spp. (10^8–10^3) ($p \leq 0.05$) was detected along with complete disappearance of some representatives of facultative bacteria, such as P*roteus*, *Citrobacter freundii*, *Enterococcus faecalis*, *Peptococcus,* and gram-positive rods were detected.

During the comparative examination of changes in the percentage composition of parietal microorganisms on the 7th day of the experiment, an increase in the percentage of *Bifidobacterium* spp. by 5.6% (from 38.3 to 43.9%) and reduction of *Lactobacillus* spp. by 31.2% (from 48.7 to 17.5%) were determined. On the 7th day, the percentage of *Bacteroides* spp. increased and was 26.3%. The percentage of *E. coli* also increased: on the 7th day, it was 0.5%. *Staphylococcus epidermidis* accounted for 0.3%. In 33% of examinations, *Vellonellaceae* was detected, the total percentage of which was 11.4% on the 7th day, and during single examinations it was up to 28%.

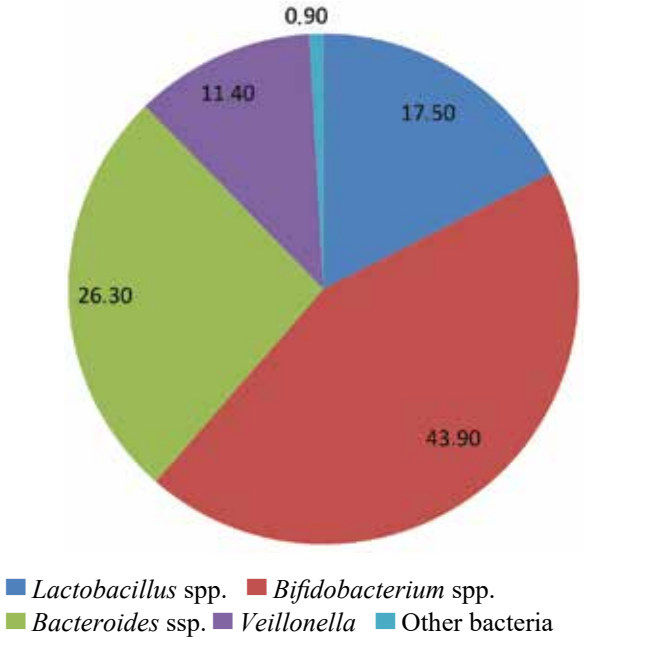

Fig.3. The percentage of the parietal microflora of the colon in rats under conditions of modeled ulcerative colitis on the 7th day

On the 10th day after modeling of ulcerative lesion in the colon wall, an increase in the total concentration of microbes up to 2.39×10^6 in compari-

son with the 7th day of the experiment and reduction of this parameter in comparison with indices of healthy animals were registered. On the 10th day, in comparison with the reference values of healthy animals, there was significant reduction in the concentration of *E. coli* (10^6–10^4), *Bifidobacterium* spp. (10^7–10^6), and *Bacteroides* spp. (10^8–10^6). Additionally, a complete absence of *Proteus mirabilis*, *Citobacter freundii*, *Enterococcus faecalis*, *Staphylococcus,* and gram-positive rod was detected. The prevailing microflora consisted of *Bacteroides* spp. (26.7%) and *Peptococcus* (27.6%), while a smaller part was represented by *Lactobacillus* spp. (17.5%), *Bifidobacterium spp.* (13.8%), and gram-positive rods (4.6%). *Bacteroides* spp. are opportunistic pathogens.

Under conditions of immunodeficiency, which develops when modeling ulcerative colitis using the original method, *Bacteroides* spp. contribute to emergence of purulent inflammation in associations with aerobic bacteria. A wide range of virulence factors, such as capsules, pilli, and outer membrane proteins, contribute to the adhesion processes. Capsular polysaccharide as an aggressive factor protects bacteria from phagocytosis. *Bacteroides* spp. produce a number of enzymes, such as neuraminidase, fibrinolysin, and heparinase, participating in invasion, as well as products of metabolism, such as short-chain fatty acids and biogenic amines, which disrupt the capacity of macrophages and leucocytes. Lipopolysaccharides take part in inactivation of the phagocytosed cells [12]. In 50% of cases, Veillonellaceae bacteria were detected, which account for 9.6% from the total bacterial count. *Veillonellaceae* are among the most common and physiologically significant bacteria of the human colon [13]. In the colon, *Veillonellaceae* are not found very often: in 1 g of feces, the content of this bacterium is in the range of 0–10^8 CFU [14]. Colitis is associated with *Veillonella parvula* (http://www.gastroscan.ru/handbook/118/4113) (Fig.4).

CONSLUSION

Quantitative and qualitative changes in the parietal microflora of the modeled ulcerative lesion of colonic mucosa in male Wistar rats are characterized by the reduction of the bacterial count by the 3rd day, development of dysbiosis due to a decrease in the concentration of *Lactobacillus* and *E. coli*, emergence of fungal microflora, the determination of opportunistic microflora and a compensatory response in the form of a significant increase in *Bifidobacterium*. However, even such a significant increase in the *Bifidobacterium* concentration on the at early stages after the surgery is not sufficient to maintain the normal balance of the intestinal microflora, which is demonstrated by significant reduction of the total parietal concentration of bacteria by the 7th day of the experiment with an increase in the absolute number of opportunistic bacteria.

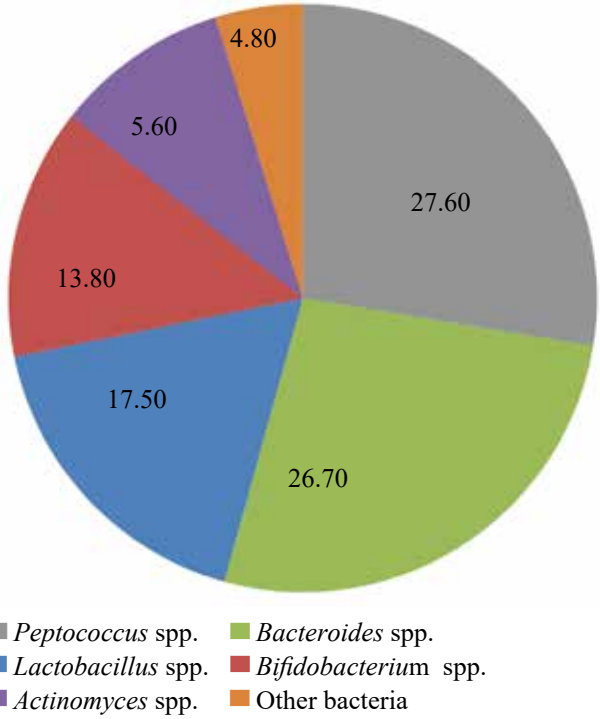

Fig. 4. The percentage of the parietal microflora of the colon in rats under condition of the modeled ulcerative colitis on the 10th day

By the 10th day of the experiment, with a small increase in the total number of parietal bacteria, the predominant microorganisms were *Bacteroides* ssp., the bacteria maintaining the inflammatory process in the colonic mucosa. *Veillonellaceae were* detected in up to 50% of cases, the presence of which is directly associated with the development of inflammation in the colon wall. Further research is obviously required both for understanding complex interaction of macro- and microorganisms and their influence on the development of pathological reactions, and for studying the possibility of correcting these conditions.

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Authors contribution

Kim A.D. – carrying out of experiments, collection of material, drafting of the manuscript. Lepekhova S.A. – conception, formulation of research aims and tasks, critical review, addition of comments and editing of the manuscript. Chashkova E.Yu. – drafting of the manuscript. Koval E.V., Fadeeva T.V., Goldberg O.A. – provision of reagents, materials, animals, and tools for calculation and analysis. Pivovarov Yu.I. – application of statistical, mathematical, computational and other methods for analysis and synthesis of the data.

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