

Analysis of the intestinal microbiome in colorectal cancer

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Abstract

Aim. To conduct a comparative analysis of the microbiome of biopsies of a tumor and normal intestinal epithelium of patients diagnosed with colorectal cancer and to identify of functional activities of the obtained bacterial isolates that affect the development of the tumor.

Methods. The study included 50 patients with malignant neoplasms of the colon: 36 men and 24 women. The mean age of the patients was 64.1±10.2 years. To analyze the microbiota of the biopsies, DNA samples were obtained from the tissue of the unaffected colon mucosa and tumor of the patients. Bacterial 16S rRNA genes fragments were amplified using bar-coded primer bakt_341f. Metagenomic next-generation sequencing was performed using the MiSeq platform (Illumina, USA). The obtained data were processed by bioinformatic methods using the QIIME package. Recognition of microorganisms depending on the morphotype and gram staining of the microflora was carried out using combination differential media and biochemical tests. Statistical analysis was carried out using Microsoft Excel, Service Pack 2 for Office XP, Statistica 6.0 (StatSoft). A comparative analysis was performed with the Student's t-test and the Mann–Whitney test in case of unmet conditions of validity. Alpha diversity of bacterial communities was quantified by the Shannon diversity index and the UniFrac distance for beta diversity analysis.

Results. In patients with colorectal cancer, 5 bacterial phyla were isolated, the predominant of which were *Firmicutes* and *Bacteroidetes*, while the content of *Actinobacteria* was low. In addition, a higher number of representatives of *Fusobacteria* was observed in the tumor tissue compared to the tissue of a healthy mucosa, at a distance of 5 centimeters proximal to the tumor. The results of this study indicate that the microbiome of a tumor and a healthy mucosa fundamentally differ from each other not only in morphotype and gram staining but also in antagonistic, hemolytic and ribonucleolytic activities.

Conclusion. Colonization of the tumor by dominant aggressive Gram-negative bacteria leads to significant changes in the tumor microbiome composition compared with normal mucosa, whose representatives are displaced from the damaged epithelium by more aggressive strains.

Keywords: colorectal cancer, microbiota, ribonuclease, functional activity of bacteria.

For citation: Gataullin B.I., Gataullin I.G., Nguyen Thi Nga, Kolpakov A.I., Ilinskaya O.N. Analysis of the intestinal microbiome in colorectal cancer. *Kazan Medical Journal*. 2021; 102 (2): 185–191. DOI: 10.17816/KMJ2021-185.

Background. Recent years have been marked by advances in molecular oncology, immunology, and microbiology, which have significantly expanded our knowledge of the carcinogenesis of colorectal cancer (CRC) and opened up new opportunities for its prevention and treatment.

For several diseases of non-microbial etiology, a complex relationship has been found with certain infections. For example, the relationship between *Helicobacter pylori* infection and the development

of stomach cancer, human papillomavirus and cervical cancer, *Salmonella typhi* and gallbladder cancer, and *Streptococcus bovis* and CRC. A change in the microbiota composition, also called dysbiosis, in feces and tissues induces a violation of the epithelial barrier function. These interactions lead to bacterial adhesion and invasion, causing impaired deoxyribonucleic acid (DNA) repair, decreased apoptosis, increased proliferation, and altered immune response to bacterial infection. The develop-

ment of CRC becomes a logical culmination of this process [1].

Other microorganisms, such as *Streptococcus pyogenes* and *Serratia marcescens*, promote growth inhibition and even the regression of colonic neoplasms [2]. Thus, the additional studies of the intestinal microflora are necessary for a better understanding of the complex interactions between the human body and its microbiota, to elucidate the specific carcinogenic mechanisms, and the development of original methods of treating CRC.

Usually, trillions of commensal bacteria coexist in the human colon, including representatives of the genera *Fusobacterium*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and widespread species of *Bacteroides fragilis*, *Escherichia coli*, *Streptococcus gallolyticus*, *Enterococcus faecalis*, and others [3]. In general, anaerobes outnumber aerobes in the intestinal bacterial community, and any disturbance in the composition of this microbiota causes a condition described as dysbiosis [4].

CRC dietary risk factors are mediated by dysbiosis of the intestinal microbiota and an imbalance of its metabolites. In this regard, bacteria associated with CRC, namely *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides fragilis*, *Escherichia coli*, and butyrate-producing bacteria, are of great interest. At recent oncology congresses, the issue of human interaction with his microbiome in colorectal carcinogenesis has been widely discussed [5–7]. In 2012, researchers used pyrosequencing of fecal bacteria to show that *Bacteroides fragilis*, *Enterococcus*, *Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus* prevailed in the intestinal microflora of patients with CRC [8].

Some bacterial taxa are associated with a lower risk of CRC. 16S rRNA sequencing has shown that *Roseburia* and other butyrate-producing bacteria of the *Lachnospiraceae* family and *Clostridium* genus are less common in patients with CRC [8]. These bacterial taxa are well-known producers of short-chain fatty acids, which serve as the preferred energy substrate for colon cells and help maintain a healthy epithelium and homeostasis [9]. In addition, butyrate has significant antitumor effects, including inhibiting tumor cell proliferation, initiating apoptosis in tumor cells, and mediating T-regulatory cell homeostasis [10, 11].

The intestinal microbiota can alter the pharmacokinetics of various drugs, including anticancer drugs, influencing the results [12–14] and/or the side effects of therapy [15, 16]. Metagenomic and metabolic profiling of the intestinal microbiota of patients can be informative for choosing a chemotherapeutic drug and predicting side effects. The therapeutic efficacy of anticancer drugs can also be

improved by actively modulating the intestinal microbiota and using probiotics, prebiotics, or specific inhibitors [17]. This approach could provide innovative “oncobiotic” strategies that combine anticancer and microbiota-targeted agents.

The mechanisms underlying the role of microflora in oncogenesis are not fully understood; however, evidence suggests that maintaining the normal intestinal microbiota is a primary measure of CRC prevention [18]. A promising direction for preventing CRC may be fecal microbiota transplantation, a procedure shown to decrease the risk of cancer in mice [19].

Additional studies of the intestinal microflora are necessary to gain a complete understanding of the complex interactions between the human body and its microbiota, elucidate the specific mechanisms of CRC carcinogenesis, and assess the contribution of bacteria to its prevention and treatment.

Aim. This study aims to conduct a comparative analysis of the microbiome of biopsies of tumor and normal intestinal epithelium of patients with diagnosed CRC and identify the functional activity of isolated bacterial isolates that affect tumor development.

Materials and methods. The study included 50 patients with malignant neoplasms of the colon, which were treated at the Republican Clinical Oncological Dispensary of the Ministry of Health of the Republic of Tatarstan from 2013 to 2018: 36 men and 24 women. The average age of the patients was 64.1 ± 10.2 years.

The malignant tumor was localized in the right half of the colon in 10 (22%) patients, in the left half of the colon in 17 (34%) patients, in the rectum in 23 (46%). Disease Stage I was diagnosed in 12% of patients, Stage II in 53%, Stage III in 25%, and Stage IV in 10%.

Patients underwent radical and palliative surgical interventions for malignant neoplasm of the colon: right hemicolectomy in 20%, left hemicolectomy in 4%, resection of the sigmoid colon in 26%, obstructive resection of the sigmoid colon in 4%, obstructive resection of the rectum in 8%, intra-abdominal rectal resection in 22%, and abdominal-perineal rectal extirpation in 16% of cases. In all patients, biopsy samples of the mucous membrane 5 cm from the tumor and tumor tissue were taken after removal of the surgical specimen (Fig. 1).

Biopsies were examined in accordance with the permission of the Ethics Committee of the Kazan State Medical Academy (Protocol No. 4 dated May 7, 2009). The resulting material was transported in sterile containers to a microbiological laboratory. Microorganisms from biopsies were washed off with sodium phosphate buffer and plated on

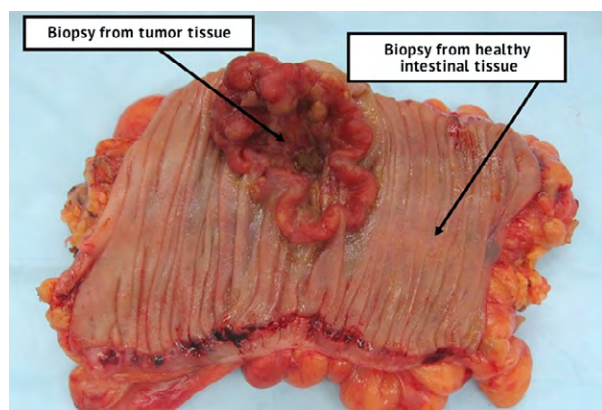


Fig. 1. Biopsy sites

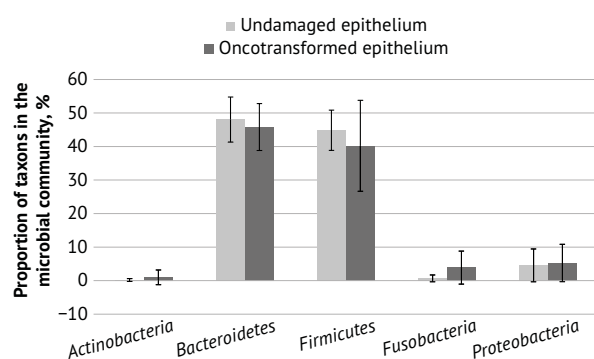


Fig. 2. The content of parietal microorganisms in the intact mucous membrane of the colon and the tumor tissue. The total number of microorganisms is taken as 100%

meat-peptone agar media and Endo medium. After 48 h, the colonies were counted, and the morphological characteristics of bacteria were determined by Gram staining. The identification of cultured microorganisms was performed by mass spectrometric analysis using MALDI-TOF Biotyper (Matrix Assisted Laser Desorption/Ionization), according to the spectrum of proteins directly from the bacterial cell without preliminary sample preparation.

DNA samples were obtained from the tissue of the unaffected colon mucosa and the tumor to analyze the microbiota of biopsy specimens. Fragments of bacterial 16S rRNA genes were amplified using the barcoded primers Bakt_341F (5'-CCT ACG GGN GGC WGC AG-3') and Bakt_805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') using high-precision DNA polymerase Phusion. The resulting amplicons for each sample were pooled and purified using Agencourt AMPure XP beads (Beckman Coulter, USA). The amount of DNA was determined using the Quant-iTdsDNA HS assay kit. New generation metagenomic sequencing was performed on the MiSeq platform (Illumina, USA). The data obtained were processed by bioinformatics methods using the QIIME software package

(an open source software package comparing and analyzing microbial communities). Data processing included assembling reads from both ends, assessing the quality of the obtained nucleotide sequences (short sequences with a read quality parameter of less than 25 were excluded from the analysis), clustering into operational taxonomic units that act as analogs of biological species (operational taxonomic units with a threshold value higher than 97%) and assigning a taxonomic status.

The studied cultures were transferred in the form of a cut-out area with agar to new Petri dishes inoculated in advance with a 6-hour broth culture of non-colicinogenic *E. coli* K12 to determine the antagonistic activity of the isolated bacteria relative to *E. coli* K12. The result was considered after 24 h of incubation at 37°C according to the zones of growth inhibition of *E. coli* K12 strain around the studied cultures. The hemolytic activity of the isolated bacteria was recorded by cultivation for 48 h at 37°C on 20% blood agar. The secreted RNase activity was determined by the formation of acid-soluble hydrolysis products from high-polymer yeast RNA introduced into an agar phosphorus-free medium by recording the size of the clear zone on the medium. If there was a clear zone around three identical colonies, the isolate was considered to be secreting RNase.

Statistical data processing was performed using Microsoft Excel, Service Pack 2 for Office XP, Statistica (StatSoft) version 6.0. The Student's *t*-test was used to conduct a comparative analysis of the data obtained, and in case of non-compliance with its conditions, the Mann-Whitney test was used. The Shannon index was used to assess the α -diversity of bacterial communities, β -diversity of the communities was assessed using the UniFrac method.

Results and discussion. The conducted microbiological analysis of biopsied intestinal epithelium, in contrast to the unstable intraluminal flora, enables examining the parietal, intestinal crypt microflora content. Microbial communities of biopsies of the normal and malignant intestinal epithelium of patients with CRC were studied by pyrosequencing of 16S rRNA gene fragments (Fig. 2).

Five bacterial phyla were isolated from the intestinal microbiocenosis of patients with CRC, the predominant of which were *Firmicutes* and *Bacteroidetes*, whereas the content of *Actinobacteria* was low (Fig. 1). In addition, a higher number of *Fusobacteria* representatives was noted in the tumor tissue compared with the healthy mucous membrane tissue.

The morphological characteristics of colonies from biopsies of unaffected mucous membranes and colon tumor tissue were used to obtain 116 bac-

Table 1. The number of isolates of the main groups of bacteria isolated from the healthy mucous membrane and tumor tissue of patients with CRC

Source of biopsy material	Bacteria				p
	Gram positive		Gram negative		
	n	%	n	%	
Healthy mucous membrane	49	80	12	20	<0.005
Tumor tissue	14	25	41	75	<0.005

Table 2. Bacterial colonization in various parts of the colon

Location	Gram-negative rods	Gram-positive rods
Right half of transverse colon	10 ⁴	10 ⁸ –10 ⁹
Left half of transverse colon	10 ⁵ –10 ⁶	10 ⁷
Rectum	10 ⁷ –10 ⁸	10 ⁶

terial isolates from patients with CRC. On average, 103 to 105 microorganisms were inoculated per one ml of the suspension obtained by washing the biopsy sample with sodium phosphate buffer (Sigma, USA).

The microflora analysis of biopsy specimens was performed using classical microbiology methods that allowed identifying those groups of bacteria characteristic for the tumor growth zone and the healthy colon mucous membrane. Sixty-one microbial isolates were isolated from the healthy tissue. Most were identified as gram-positive microorganisms (80% of observations) (Table 1), primarily facultative anaerobic and anaerobic microorganisms of the *Lactobacillaceae* and *Bifidobacteriaceae* families. Fifty-five microbial isolates were isolated from tumor tissue, of which gram-negative bacteria prevailed (75% of observations) (Table 1), which included the *Fusobacterium* genus. In most cases, gram-negative bacteria belonged to the *Enterobacteriaceae* family, an opportunistic facultative anaerobic bacteria. Note that the *Fusobacterium* genus is distinguished by its ability to secrete phospholipase A, which facilitates the invasion of bacteria into deep tissues, and leukocidin, which exhibits a cytotoxic effect on various cells.

In addition, opportunistic pathogens were identified in the tumor tissue - *Staphylococcus aureus* (*S. aureus*), *Klebsiella* (*Klebsiella*), and *Proteus* (*Proteus*), which have pronounced antagonistic properties.

Quantitative analysis of the microflora of the colon demonstrates the increase in the number of opportunistic microflora of the colon in the direction

from the head of the cecum to the sphincter ani externus and the corresponding decrease in the number of gram-positive lactic acid bacteria (Table 2).

Hemolytic, ribonucleolytic, and antagonistic activities of microorganisms are the most important factors in the competitive struggle of bacteria for colonization of the epithelium.

Bifidobacteria have high antagonistic activity against pathogenic and opportunistic microorganisms due to the synthesis of organic fatty acids; lactobacilli inhibit the growth of putrefactive microorganisms due to the synthesis of lactic acid; and *E. coli* produce colicins, antibiotic-like substances, that inhibit the growth of enteropathogenic intestinal [20].

The highest concentration of microorganisms with antagonistic properties was found on the mucous membrane adjacent to the tumor, that is, in the zone of active competitive struggle of microorganisms. We paid particular attention to determining the activity of extracellular RNases based on data on the cytotoxic effect of colicin E3 on tumor cells which serves as a highly specific RNase and a potential anticancer agent [21]. Some other bacterial RNases [22] also have antitumor and anti-immigration effects, which can also prevent CRC development.

We found that after eight h of cultivation on an RNA-enriched, phosphorus-deficient, synthetic medium, 83% of isolates of oncotransformed epithelium and 27% of isolates of conventionally healthy epithelium had significant extracellular ribonuclease activity. After 24 h of cultivation, 91% of bacterial isolates of healthy epithelial tissues showed the same low level of activity. Nine percent of isolates of healthy epithelium did not show any visible extracellular RNase activity. By combining the isolates according to Gram staining, both groups of bacteria could synthesize extracellular RNases, the activity of which increased with the time of cultivation (Table 3). The activation of the synthesis of extracellular RNases in groups of bacteria colonizing damaged intestinal tissue areas can be viewed as a possible control mechanism by the normal microflora to counteract colorectal tumor formation.

The results obtained indicate that the microbiome of a tumor and a healthy mucous membrane is fundamentally different in morphotype, and Gram affiliation, and antagonistic, hemolytic, and ribonucleolytic activity.

Conclusions. Thus, we established that bacteria colonizing colon tumor tissue have a more pronounced antagonistic activity than the unaffected mucous membrane since they can synthesize secreted ribonucleases. The colonization of a tumor by dominant gram-negative bacte-

Table 3. Percentage of bacterial isolates from biopsies of oncotransformed and normal rectal epithelium with ribonuclease activity

The proportion of bacteria with secreted RNases, percent of the total number of isolates of a certain Gram affiliation*				
Culturing time of bacteria on a synthetic phosphorus-free medium, h	8		24	
Gram affiliation	+	–	+	–
Epithelium type				
Conditionally healthy	50	24	75	93
Malignant	100	81.6	100	100
p	<0.001	<0.001	<0.05	<0.05

Note: * the total number of isolates of each Gram affiliation is taken as 100%

ria with pronounced aggressive properties causes significant changes in the composition of the tumor microbiome compared with the healthy mucous membrane. These changes include displacing representatives from the damaged epithelium with more aggressive neighbors.

The high level of ribonucleolytic activity of tumor microorganisms indicates that they contribute to eliminating tumor cells. Multidirectional metabolic processes occur in the microbiome of tumor tissue, activating and inhibiting its growth, indicating poorly studied adaptation mechanisms that allow microflora to resist oncological diseases.

CONCLUSIONS

1. The analysis of the microflora of biopsies taken from patients with CRC showed that the intact epithelium is colonized mainly by gram-positive bacteria that do not possess antagonistic and hemolytic activity are capable of producing secreted ribonucleases.

2. The tumor tissue biopsy specimens are mainly seeded with gram-negative microorganisms with pronounced antagonistic and hemolytic properties.

3. The study results of the intestinal tissue microbiome in CRC suggest that identifying critical bacteria and their metabolites in the tumor will become valuable prognostic disease markers.

Author contributions. B.I.G., N.T.N., A.I.K. conducted research; I.G.G. and O.N.I. were responsible for analyzing the results and supervising the work.

Funding. The study had no external funding.

Conflict of interest. The authors declare that they have no conflicts of interest regarding the presented article.

REFERENCES

1. Park C.H., Eun C.S., Han D.S. Intestinal microbiota, chronic inflammation, and colorectal cancer. *Intest. Res.* 2018; 16 (3): 338–345. DOI: 10.5217/ir.2018.16.3.338.
2. Emelyanova M.A., Amosenko F.A., Semyanikhina A.V., Aliev V.A., Barsukov Yu.A., Lyuchenko L.N., Nasedkina T.V. Detection of somatic mutations in kras, braf and pik3ca genes in colorectal cancer patients using biochips. *Molekulyarnaya biologiya.* 2015; 49 (4): 617–627. (In Russ.) DOI: 10.7868/S0026898415040035.
3. Malikhova O.A., Karasev I.A., Davydkina T.S., Vereshchak V.V., Malikhov A.G., Tumanyan A.O. Intestinal microbiome and colorectal cancer. literature review. *Povolzhskiy onkologicheskiy vestnik.* 2019; 10 (4): 45–51. (In Russ.)
4. Neish A.S. Microbes in gastrointestinal health and disease. *Gastroenterology.* 2009; 136 (1): 65–80. DOI: 10.1053/j.gastro.2008.10.080.
5. Park C.H., Eun C.S., Han D.S. Intestinal microbiota, chronic inflammation, and colorectal cancer. *Intest. Res.* 2018; 16 (3): 338–345. DOI: 10.5217/ir.2018.16.3.338.
6. Hughes L.A., Simons C.C., van den Brandt P.A., van Engeland M., Weijenberg M.P. Lifestyle, diet, and colorectal cancer risk according to (epi)genetic instability: current evidence and future directions of molecular pathological epidemiology. *Curr. Colorectal. Cancer Rep.* 2017; 13: 455–469. DOI: 10.1007/s11888-017-0395-0.
7. Ilinskaya O.N., Kharitonova M.A., Doinicova A.N. Analyzing the age diversity of patients with colorectal cancer. *Intern. J. Pharmac. Res.* 2020; 12 (1): 768–774. DOI: 10.31838/ijpr/2020.12.01.149.
8. Wang T., Cai G., Qiu Y., Fei N., Zhang M., Pang X., Jia W., Cai S., Zhao L. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J.* 2012; 6: 320–329. DOI: 10.1038/ismej.2011.109.
9. Hamer H.M., Jonkers D., Venema K., Vanhoutvin S., Troost F.J., Brummer R.-J. Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* 2008; 27: 104–119. DOI: 10.1111/j.1365-2036.2007.03562.x.
10. Ruemmele F.M., Schwartz S., Seidman E.G., Dionne S., Levy E., Lentze M.J. Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. *Gut.* 2003; 52: 94–100. DOI: 10.1136/gut.52.1.94.
11. Smith P.M., Howitt M.R., Panikov N. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science.* 2013; 341: 569–573. DOI: 10.1126/science.1241165.
12. Espín J.C., González-Sarriás A., Tomás-Barberán F.A. The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. *Biochem. Pharmacol.* 2017; 139: 82–93. DOI: 10.1016/j.bcp.2017.04.033.
13. Guthrie L., Gupta S., Daily J., Kelly L. Human microbiome signatures of differential colorectal cancer drug metabolism. *NPJ. Biofilms. Microbiomes.* 2017; 3: 27. DOI: 10.1038/s41522-017-0034-1.
14. Geller L.T., Barzily-Rokni M., Danino T., Jonas O.H., Shental N., Nejman D. Potential role of intratumor bacteria in

mediating tumor resistance to the chemotherapeutic drug gemcitabine. *Science*. 2017; 357: 1156–1160. DOI: 10.1126/science.aah5043.

15. Wallace B.D., Wang H., Lane K.T., Scott J.E., Orans J., Koo J.S., Venkatesh M., Jobin C., Yeh L.A., Mani S., Redinbo M.R. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. *Science*. 2010; 330: 831–835. DOI: 10.1126/science.1191175.

16. Nguen N.G., Vafin R.R., Rzhanova I.V., Kolpakov A.I., Gataullin I.G., Tyul'kin S.V., Sinyagina M.N., Grigor'eva T.V., Il'inskaya O.N. Molekulyarno-geneticheskij analiz mikroorganizmov s vnutriepitelial'noj invaziej, vydelennyh ot bol'nyh kolorektal'nym rakom. *Molekulyarnaya genetika, mikrobiologiya, virusologiya*. 2016; (1): 13–18. (In Russ.) DOI: 10.18821/0208-0613-2016-34-1-13-18.

17. Kuczma M.P., Ding Z.C., Li T., Habetsion T., Chen T., Hao Z., Bryan L., Singh N., Kochenderfer J.N., Zhou G. The impact of antibiotic usage on the efficacy of chemoimmunotherapy is contingent on the source of tumor-reactive T cells. *Oncotarget*. 2017; 8: 111 931–111 942. DOI: 10.18632/oncotarget.22953.

18. Mulendeev S.V., Soloviev I.A., Shostka K.G., Arutyunyan K.V., Roma L.D. The role of intestinal dysbiosis in co-

lorectal cancer etiology and prevention (scientific review). *Profilakticheskaya i klinicheskaya meditsina*. 2017; (4): 55–60. (In Russ.)

19. Kochkina S.O., Gordeev S.S., Mamedli Z.Z. Role of human microbiota in the development of colorectal cancer. *Tazovaya hirurgiya i onkologiya*. 2019; 9 (3): 11–17. (In Russ.) DOI: 10.17650/2686-9594-2019-9-3-11-17.

20. Eklöf V., Löfgren-Burström A., Zingmark C., Edin S., Larsson P., Karling P., Alexeyev O., Rutegård J., Wikberg M.L., Palmqvist R. Cancer-associated fecal microbial markers in colorectal cancer detection. *Int. J. Cancer*. 2017; 141: 2528–2536. DOI: 10.1002/ijc.31011.

21. Zelenikhin P.V., Mohamed I.S.E., Nadyrova A.I., Sirotkina A.A., Ulyanova V.V., Mironova N.L., Mitkevich V.A., Makarov A.A., Zenkova M.A., Ilinskaya O.N. Bacillus pumilus ribonuclease inhibits migration of human duodenum adenocarcinoma hutu 80 cells. *Molekulyarnaya biologiya*. 2020; 54 (1): 146–152. (In Russ.) DOI: 10.31857/S0026898420010176.

22. Soelaiman S., Jakes K., Wu N., Li C., Shoham M. Crystal structure of colicin E3: implications for cell entry and ribosome inactivation. *Mol. Cell*. 2001; 8 (5): 1053–1062. DOI: 10.1016/s1097-2765(01)00396-3.