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Е. Г. Малаева*, И. О. Стома

Гомельский государственный медицинский университет, Гомель, Республика Беларусь

МИКРОБИОТА И ДОЛГОСРОЧНЫЙ ПРОГНОЗ ПРИ ЦИРРОЗЕ ПЕЧЕНИ

E. G. Malaeva*, I. O. Stoma

Gomel State Medical University, Gomel, Republic of Belarus

Microbiota and Long-Term Prognosis in Liver Cirrhosis

Резюме

Цель. Провести сравнение микробиоты кишечника у пациентов с анамнезом цирроза печени менее и более 10 лет. **Материалы и методы.** Проведено одномоментное исследование и метагеномное секвенирование кала 40 госпитализированных пациентов с циррозом печени, из них 35 — с анамнезом цирроза менее 10 лет и 5 — более 10 лет. Высокопроизводительное секвенирование проводилось с использованием генетического анализатора MiSeq (Illumina, США) и протокола, основанного на анализе вариабельных регионов гена 16s рРНК. Исследование зарегистрировано в Clinicaltrials.gov (NCT05335213). Анализ данных проводили с использованием алгоритма Kraken2. Анализ различия пропорционального состава микробиома между группами осуществлялся с помощью полиномиального моделирования Дирихле (Likelihood-Ratio-Test Statistics: Several Sample Dirichlet-Multinomial Test Comparison), теста Манна-Уитни с предварительным преобразованием данных методом CLR-преобразования (Centered log ratio transform), дифференциального анализа экспрессии генов на основе отрицательного биномиального распределения (DEseq2). Уровень значимости α принят равным 0,05. **Результаты.** У пациентов с циррозом печени доминирующими филотипами микробиоты кала являются *Firmicutes*, *Bacteroidetes, Proteobacteria*, *Actinobacteria*, к минорным компонентам относятся таксоны *Aquificae*, *Coprothermobacterota*, *Tenericutes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Thermotogae*, *Chlorobi, Candidatus Saccharibacteria, Synergistetes.* Установлены значимые различия плотности доминирующих и минорных филотипов кишечных бактерий, таких как *Actinobacteria, Proteobacteria, Coprothermobacterota, Candidatus Saccharibacteria*, *Synergistetes,* а также некоторых классов, родов, видов бактерий у пациентов с разной продолжительностью заболевания ($p < 0,05$). **Заключение.** Не вызывает сомнения влияние кишечной микробиоты на компенсацию функций печени. Установленные различия композиционного состава микробиоты у пациентов с циррозом печени в зависимости от выживаемости на протяжении 10 лет имеют научное и практическое значение для формирования научно-обоснованного подхода применения микробиом-ассоциированных интервенций.

Ключевые слова: *цирроз печени, микробиота, филотипы бактерий*

Конфликт интересов

Авторы заявляют, что данная работа, её тема, предмет и содержание не затрагивают конкурирующих интересов

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Abstract

Purpose. To compare the gut microbiota in patients with an anamnesis of liver cirrhosis of less than and more than 10 years. **Materials and methods.** A one-stage study and metagenomic fecal sequencing of 40 hospitalized patients with liver cirrhosis were conducted, of which 35 were with a history of cirrhosis of less than 10 years and 5 — more than 10 years. High-throughput sequencing was performed using a MiSeq genetic analyzer (Illumina, USA) and a protocol based on analysis of 16s rRNA gene variable regions. The study was registered in Clinicaltrials.gov (NCT05335213). Data analysis was performed using Kraken2 algorithm. The analysis of the difference in the proportional composition of the microbiome between the groups was carried out using polynomial Dirichlet modeling (Likelihood-Ratio-Test Statistics: Several Sample Dirichlet-Multinomial Test Comparison), the Mann-Whitney

*Контакты: Екатерина Геннадьевна Малаева, e-mail: dr-malaeva@mail.ru
*Contacts: Ekaterina G. Malaeva, e-mail: dr-malaeva@mail.ru
ORCID ID: https://orcid.org/0000-0003-1051-0787

test with preliminary data transformation by CLR transformation (Centered log ratio transform), differential analysis of gene expression based on negative binomial distribution (DESeq2). The significance level α assumed to be 0.05. **Results.** In patients with liver cirrhosis, the dominant phylotypes of fecal microbiota are *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*, minor components include taxa *Aquificae*, *Coprothermobacterota*, *Tenericutes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Thermotogae*, *Chlorobi.* Significant differences have been established in the density of dominant and minor philotypes of gut bacteria, such as *Actinobacteria, Proteobacteria, Tenericutes, Coprothermobacterota*, as well as some classes, genera, bacterial species in patients with different disease duration ($p < 0.05$). **Conclusion.** There is no doubt about the effect of gut microbiota on compensation for liver function. The established differences in the composition of the microbiota in patients with liver cirrhosis depending on survival over 10 years are of scientific and practical importance for the formation of an evidence-based approach to the use of microbiome-associated interventions

Key words: *liver cirrhosis, microbiota, bacterial phylotypes*

Conflict of interests

The authors declare no conflict of interests

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HC — hepatic cirrhosis

Introduction

Hepatic cirrhosis (HC) is a common terminal stage of chronic hepatic diseases and is associated with a cascade of events, including excessive bacterial growth in the intestine and disbacteriosis. Bacterial toxins, entering portal or systemic blood flow, can directly kill hepatic cells, while disbacteriosis also affects the barrier function of the intestine and enhances bacterial translocation, leading to infections, systemic inflammation and vasodilatation, which contribute to acute decompensation and organ failure. Different microbiota composition can impact the rate of complications, disease prognosis and survivability of patients [1, 2].

A number of studies identified cirrhosis-specific microbiota profiles, where *Fusobacteria, Proteobacteria, Enterococcaceae* and *Streptococcaceae* prevail, with questionable reduction in *Bacteroidetes, Ruminococcus, Roseburia, Veillonellaceae* and *Lachnospiraceae*, irrespective of cirrhosis aetiology [1, 2, 3]. In addition to an increase in the number of pathogenic taxon, HC is associated with fewer potentially useful taxons, such as *Akkermansia* [1].

Genetic abundance of microbes in faeces, microbial density and the diversity of species reduce in patients with decompensated cirrhosis vs. compensated HC [4]. A significant drop in the number of faecal *Clostridiales XIV, Ruminococcaceae* and *Lachnospiraceae*, with a marked increase in pathogenic taxons, such as *Enterococcaceae, Staphylococcaceae* and *Enterobacteriaceae*, was reported in patients with decompensated cirrhosis [2]. Metagenomic sequencing was used to isolate *Alistipes indistinctus, Bilophila wadsworthia, Bilophila sp. 4_1_30, Ruminococcus champanellensis, Tannerella sp. 6_1_58FAA_CT1, Clostridium botulinum, Clostridium leptum, Clostridium methylpentosum, and Clostridium sp. MSTE9*, from faeces, the concentration of which was lower, whereas faeces abundance with *Veillonella atypica, Veillonella sp. ACP1, Veillonella dispar, and Veillonella sp.* was higher in patients with decompensated cirrhosis vs. compensated hepatic cirrhosis [1].

Traditionally, progression from compensated to decompensated hepatic cirrhosis was treated as the point of no return in the natural course of the disease. However, this point of view has been questioned by recent data on disease regression and hepatic function recompensation when the primary condition is suppressed/cured. In order to develop a uniform definition of recompensated hepatic cirrhosis, Baveno VII Consensus established standardised criteria, which include elimination of the primary causative factor and any decompensating events, as well as sound improvement of liver functions. An initial idea of hepatic recompensation was based on previous studies, which demonstrated that cure/suppression of the primary cause in patients with previous decompensation results in significant clinical improvements and favourable outcomes and can even exclude candidates for liver transplantation [5, 6]. An impact by intestinal microbiota on hepatic disease regression and liver function recompensation is of little doubt, and it increases the life expectancy of patients. This new research trend in hepatology is promising and practice-oriented.

Changes in intestinal microbiome are associated with poorer 5-year life expectancy in HC, and it has been proven by Russian scientists [7]. Of interest is the study both of dominant and minor taxons of faecal microbiota in HC, including patients with a long-term expectancy of over 10 years, and search for new biomarkers [8].

Purpose

To compare intestinal microbiota in patients with a history of hepatic cirrhosis of less than and more than 10 years.

Materials and Methods

Adult patients, admitted to the Gastroenterology Unit of the State Healthcare Institution Gomel City Clinical Hospital No. 3 (Republic of Belarus) with confirmed hepatic cirrhosis in 2022–2023, were included in the protocol of collection and deep-freezing of faeces samples. The study protocol was approved by the Ethics Committee at the Gomel State Medical University (Minutes No. 4 dated September 30, 2021). The study was registered at Clinicaltrials.gov (NCT05335213).

Metagenomic sequencing was performed for 40 faeces samples from HC patients, including 35 patients with a history of cirrhosis of less than 10 years and 5 patients — over 10 years. Inclusion criteria: over 18 years old; HC confirmed by clinical, laboratory, imaging and/or morphological data. Exclusion criteria: antibacterial therapy within a month before the study; autoimmune diseases, cancer, HIV infection, organ transplants.

Samples for metagenomic sequencing were collected in the morning using special dry sterile vials and disposable sterile scapula. Samples were transported to the laboratory within 1–2 hours after collection, in a transport container with the temperature of +2...+8°C. Faeces and urine samples were stored at -80°C. Faeces and urine samples were thawed for DNA extraction for metagenomic sequencing at room temperature [9].

High-performance sequencing was conducted using genetic analyser MiSeq (Illumina, USA) under a protocol involving analysis of variable regions of gene 16s of ribosomal RNA. Data were analysed with Kraken2 algorithm. Primer sequences were removed with the help of Preprocess 16S (https://github.com/masikol/preprocess16S), low-quality reads — with Trimmomatic. Statistical values were calculated using statistical software R (version 4.2.1), library tidyverse (version 1.3.1) and packages rstatix (version 0.7.0), HMP (version 2.0.1), DESeq2 (version 1.37.4), ANCOMBC (version 1.99.1), ggpubr (version 0.4.0), phyloseq (version 1.41.0), datawizard (version 0.4.1), microbiome (version 1.19.0), vegan (version 2.6-2).

Results were described with standard methods of descriptive statistics. Differences in microbiome composition were analysed using various methods: Mann — Whitney U test with CLR-transformation (centred log ratio transformation); differential analysis of gene expression based on negative binomial distribution (DEseq2); microbiome composition analysis with offset correction (ANCOM-BC). Differences between proportional microbiome composition between groups were analysed using Dirichlet multinomial modelling (Likelihood-Ratio-Test Statistics: Several Sample Dirichlet-Multinomial Test Comparison).

Biodiversity indices were Shannon's, Simpson's and Chao1 indices. Beta-diversity was analysed using the Principal Coordinate Analysis (PCoA) method; a measure of distance was Bray — Curtis index. Permutation multivariate analysis of variance (PERMANOVA) was used to analyse significance of differences between groups. The significance level α was 0.05.

Results

The study included 22 men and 18 women; mean age was 51.9 years old. 23 patients had alcoholic HC, 8 — of undefined origin, 9 — mixed origin (HCV + alcohol consumption). In all patients, the disease manifested as hepatic encephalopathy; 29 patients had ascites; HC in 6 patients was complicated with bleeding from varicose veins of the oesophagus. Based on the purpose of the study, all patients were divided into groups: a history of CP of less than and more than 10 years (Table 1).

Table 1. *Clinical and demographic basic characteristics of patients with liver cirrhosis (LC)*

Parameters (for quantitative — Me)	Groups		p-value
	LC up to 10 years n=35	LC more than 10 years n=5	
Gender, m/f	18/17	4/1	0,47
Age, years	49,5	68,6	0,003
Total bilirubin, mkmol/l	128,4	38,8	0,03
Albumin, g/l	28,6	37	0,005
The prothrombin index	64,5	81,6	0,03
Urea, mmol/l	8,5	7,46	0,75
Creatinine, mmol	109,2	96,2	0,75
HE, stage I-II/III-IV	35/0	5/0	0,99
Ascites, –/+	6/29	5/0	0,0007
Varices bleeding, –/+	29/6	5/0	0,99
Severity class A+B/C	7/28	4/1	0,02

Note: HE — hepatic encephalopathy

Of note, the degree of hepatic function compensation was higher in patients, who had the disease for over 10 years, as seen by bilirubin, albumin, prothrombin levels.

In HC patients, dominant phenotypes of faecal microbiota are *Firmicutes* (median density: over 50 %), *Bacteroidetes* (median density: over 38 %), *Proteobacteria*, *Actinobacteria*; minority components include *Aquificae*, *Coprothermobacterota*, *Tenericutes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Thermotogae*, *Chlorobi* (median relative representation: less than 0.05 %, but over 0.005 %) (Figure 1).

The alpha diversity index of intestinal microbiota in patients with HC with various disease duration is characterised with lack of marked differences. However, beta diversity parameters, which indicate differences between ecosystems by showing the degree of difference of one community from another, tend to have different taxonomic composition between groups of patients with cirrhosis with survivability of less than and more than 10 years (p = 0.067).

The Dirichlet multinomial parameter test for the differences between the overall microbiome composition between faeces samples of patients with differing long-term prognosis demonstrated no differences ($\chi dc = 6.62$, p = 0.25) (Figure 2).

Results of comparison of the relative representation of dominant taxons in the groups are presented in Table 2.

The results of this test allowed identifying significant differences in the density of dominant phylotypes *Actinobacteria* and *Proteobacteria* in faeces of patients with various disease duration. Representatives of taxon *Proteobacteria* are involved in bacterial translocation processes and are associated with complications in HC [7]; their concentration prevails if disease duration is less than 10 years vs. patients with a long-lasting disease and favourable long-term prognosis (p = 0.047). At the same time, phylotype *Actinobacteria* diversity, including *Bifidobacterium* sp., is higher in patients with survivability of over 10 years (p = 0.01). Similar results were obtained using the method for analysing differential representation DESeq2 with included covariates: sex, severity and age (Figure 3).

In addition to the above, significant differences in the density of minor phylotypes of faecal microbiota have been identified, and it has been established that, in patients with HC duration of less than 10 years, *Coprothermobacterota* and *Candidatus Saccharibacteria* prevail, while for disease duration of over 10 years, *Synergistetes is prevailing.* Since a majority of bacteria have been discovered quite recently and have not been cultured yet, it is impossible to define their function in the human body.

Later, a study of significant differences in the density of bacteria from certain classes, geni and species in patients in both groups was conducted.

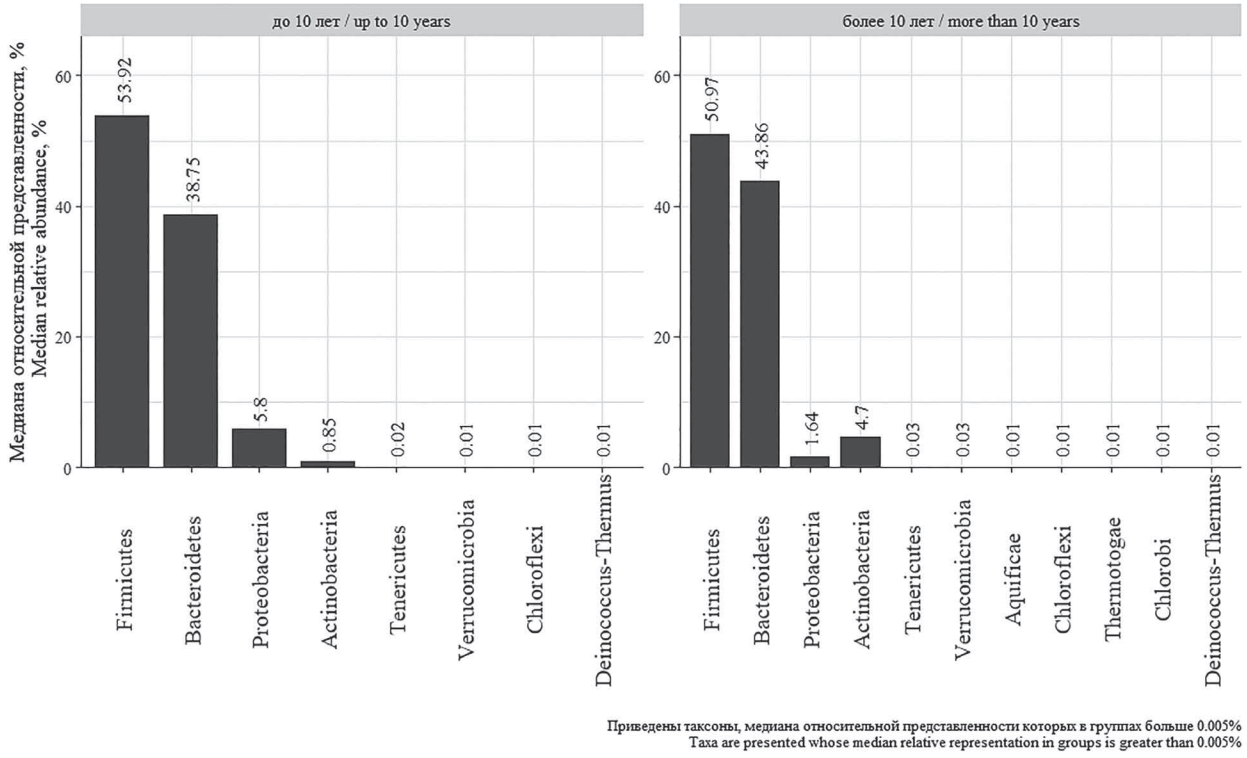

Figure 1. *Density and diversity of intestinal microbiota phylotypes with liver cirrhosis duration up to 10 years (left) and more than 10 years (right)*

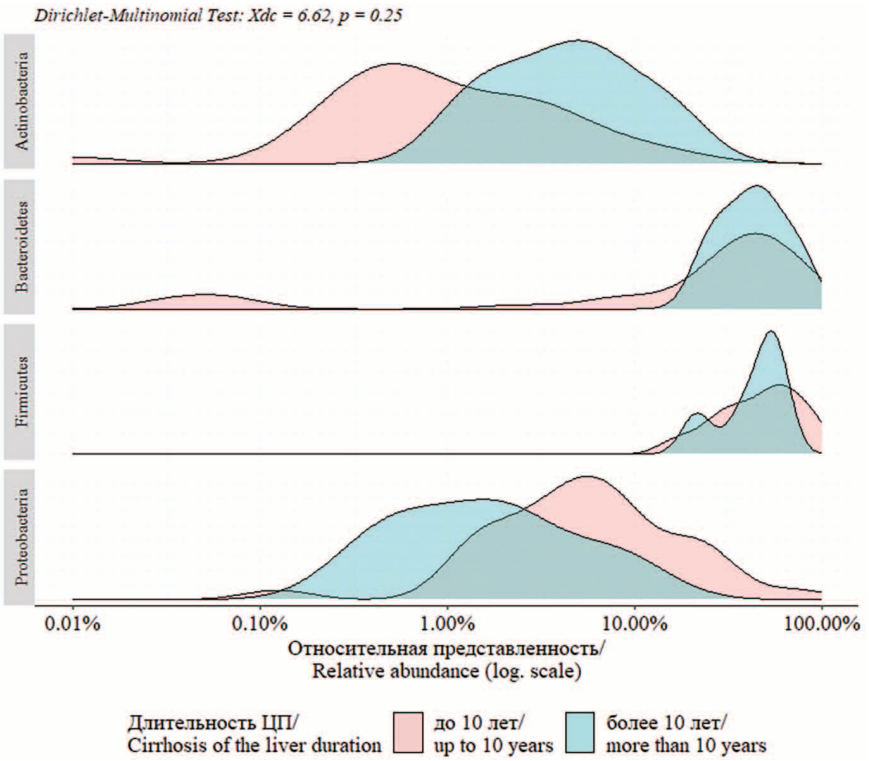

Figure 2. *Graphs of the nuclear density distribution of the relative representation of the most numerous taxa in cirrhosis of the liver duration up to 10 years and more than 10 years*

Table 2. *Comparison of the density of dominant fecal phylotypes in patients with liver cirrhosis duration less than and more than 10 years. Mann-Whitney Test*

Phylum	LC up to 10 years Me (LQ;UQ), %	LC more than 10 years Me (LQ;UQ), %	p
Actinobacteria	0,85 (0,40; 2,39)	4,70 (2,45; 6,73)	0,01
Bacteroidetes	38,75 (14,41; 53,21)	43,86 (33,01; 52,48)	0,40
Firmicutes	53,92 (32,31; 67,74)	50,97 (37,73; 55,60)	0,63
Proteobacteria	5,80 (2,73; 9,52)	1,64 (0,73; 2,55)	0,047

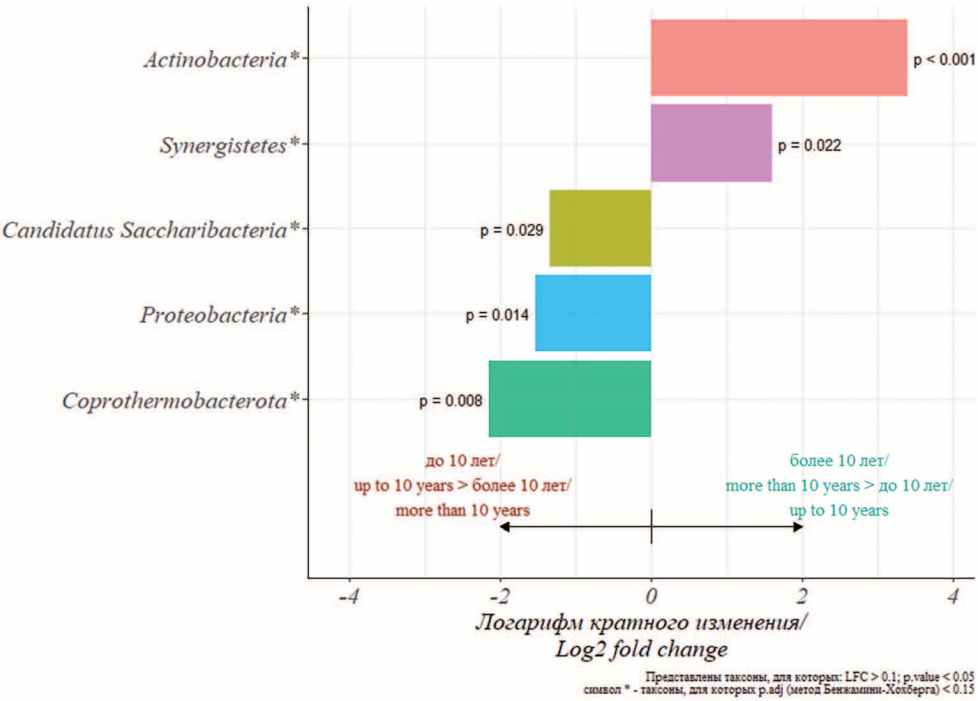

Figure 3. *Analysis of the differential representation of fecal taxa at the phylotype level in cirrhosis of the liver duration up to 10 years (left) and more than 10 years (right). DESeq2 method*

The most common classes of bacteria in faecal microbiota of HC patients are *Bacteroidia* (over 35 %), *Clostridia* (over 24 %), *Bacilli*, *Actinomycetia*, *Negativicutes*, *Gammaproteobacteria*, *Coriobacteriia*, *Erysipelotrichia*.

Patients with cirrhosis and various long-term survivability have marked differences in the density of bacterial classes *Actinomycetia, Coriobacteriia, Synergistia, Opitutae, Coprothermobacteria*, *Epsilonproteobacteria*, *Betaproteobacteria* (Figure 4).

The most common geni of bacteria in faeces of HC patients are *Prevotella*, *Faecalibacterium, Bifidobacterium, Lachnospira, Roseburia, Ruminococcus, Streptococcus, Bacteroides, Blautia.* HC patients with various long-term disease prognosis have different densities in geni of faecal bacterial taxons, such as *Anaerobutyricum*, *Anaerostipes*, *Bifidobacterium*, *Coprococcus*, *Dialister* (Table 3).

Currently, it is assumed that representatives of *Bifidobacterium* have favourable effect on human health. They are widely used as probiotics in the management of numerous GIT conditions, especially *Bifidobacterium longum*, *Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium animalis,* and *Bifidobacterium adolescentis*. In HC patients, whose disease lasts for over 10 years, faecal abundance of *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* is significantly higher than in patients with disease duration of less than 10 years (Figure 5).

Intervention studies of various medicinal products and therapies caused an increase in the number of *Bifidobacterium* spp. and better clinical outcomes in patients with hepatic diseases, thus confirming viability of microbiota modifications in patients with HC [10, 11].

Abundance of *Veillonella parvula* in faeces of HC patients is associated with infection development because of weak immunity in cirrhosis, since this bacterium is common in patients with infective endocarditis, meningitis, osteomyelitis. In a long-lasting disease,

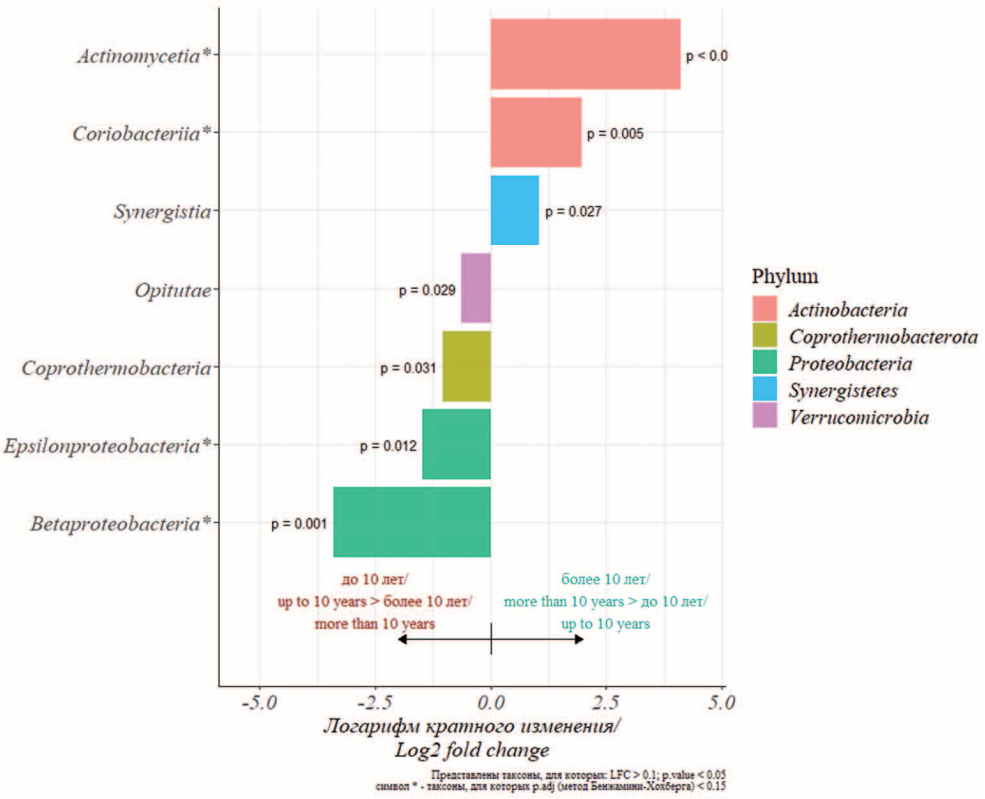

Figure 4. *Analysis of the differential representation of classes of fecal bacteria in cirrhosis of the liver duration up to 10 years (left) and more than 10 years (right). The DESeq2 method with the covariants gender, severity class and age included in the model*

Table 3. *Comparison of the density of fecal bacterial genera in patients with liver cirrhosis duration less than and more than 10 years. Mann-Whitney Test*

Genus	LC up to 10 years Me (LQ;UQ), %	LC more than 10 years Me (LQ;UQ), %	p
Anaerobutyricum	4,90 (3,19; 5,73)	6,45 (6,08; 6,53)	0,003
Anaerostipes	5,24 (4,25; 6,39)	6,55 (6,47; 6,56)	0,02
Bifidobacterium	5,27 (4,00; 6,49)	7,99 (7,95; 8,33)	0,03
Coprococcus	6,05 (4,64; 6,68)	7,61 (6,94; 7,76)	0,008
Dialister	4,74 (3,14; 5,48)	6,72 (5,79; 7,24)	0,04

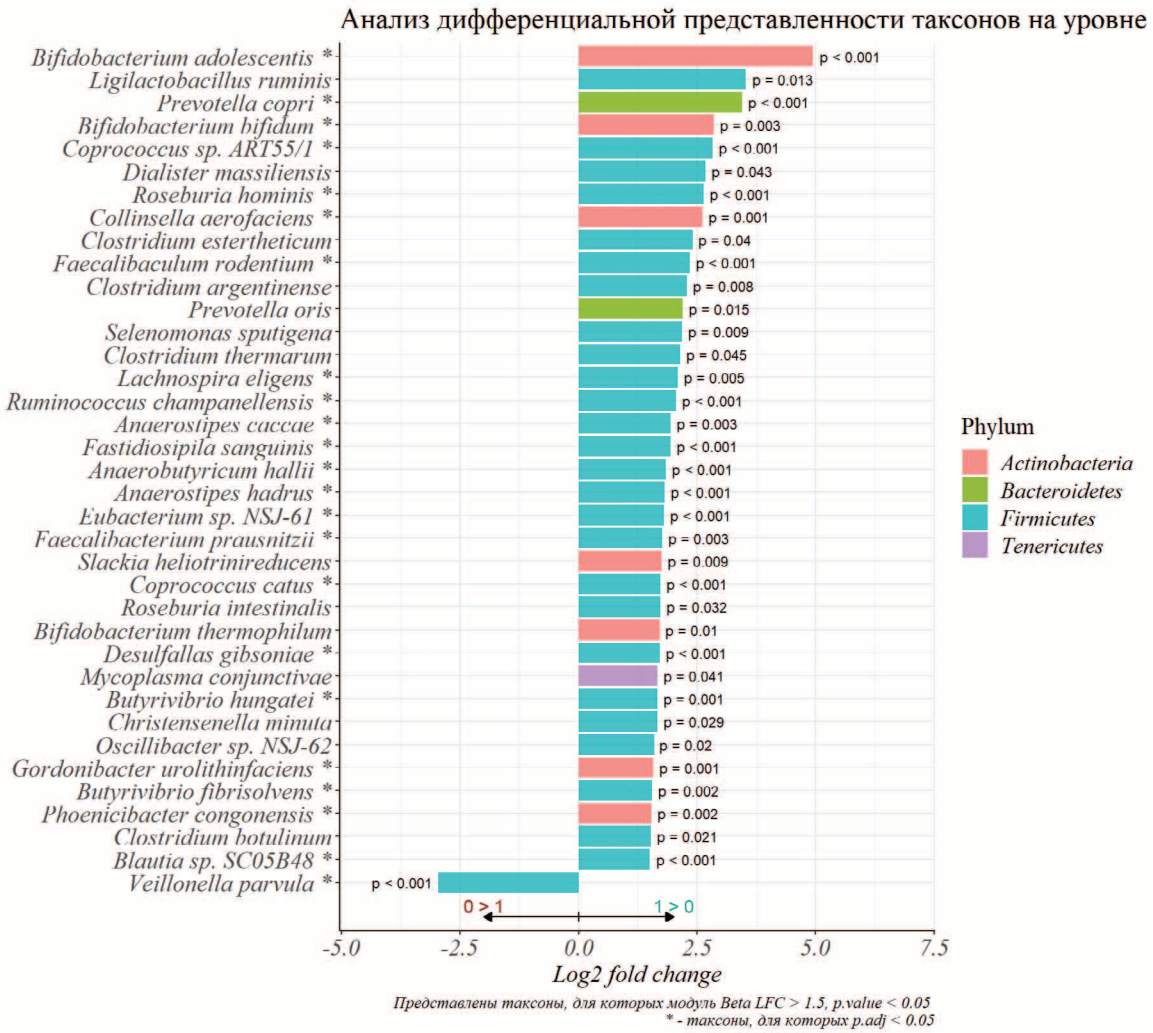

Figure 5. *Analysis of the differential representation of fecal bacterial species in liver cirrhosis duration up to 10 years (left) and more than 10 years (right). ANCOM-BC model*

faeces of patients are rich in *Faecalibacterium prausnitzii, Anaerobutyricum hallii, Anaerostipes hadrus, Prevotella copri, Enterococcus hirae, Roseburia hominis, Faecalibaculum rodentium* ***spp.,*** which are involved in synthesis of short-chain fatty acids, intestinal barrier strengthening, supporting local immunity of gut lining and other protective functions [12]. *Faecalibacterium prausnitzii* is an anti-inflammatory bacterium, which stimulates interleukin-10 production and inhibits expression of interleukin-12 and interferon gamma. *Ruminococcaceae, Lachnospiraceae and Faecalibacterium prausnitzii* are bacteria producing butyrate, which is an important source of energy for enterocytes and impact the barrier function of the intestine by stimulating close bonds and mucus production. Therefore, a unique composition of intestinal microbiota can have both protective role in the natural course of HC and hepatic function compensation and, on the contrary, can be a factor which affects disease progression, decompensation, development of associated complications and patient survivability [13–15].

Conclusion

The composition of intestinal microbiota in patients with HC depends on the long-term survivability of over 10 years. In patients with cirrhosis, dominant phylotypes of faecal microbiota are *Firmicutes* (median density: over 50 %), *Bacteroidetes* (median density: over 38 %), *Proteobacteria*, *Actinobacteria*; minor component include the following taxons: *Aquificae*, *Coprothermobacterota*, *Tenericutes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Thermotogae*, *Chlorobi, Candidatus Saccharibacteria*, *Synergistetes*. The most common classes of bacteria are *Bacteroidia* (over 35 %), *Clostridia* (over 24 %), *Bacilli*, *Actinomycetia*, *Negativicutes*, *Gammaproteobacteria*, *Coriobacteriia*, *Erysipelotrichia*. The most common geni of intestinal bacteria are *Prevotella*, *Faecalibacterium, Bifidobacterium, Lachnospira, Roseburia, Ruminococcus, Streptococcus, Bacteroides, Blautia.* In HC patients with disease duration of over 10 years, faecal microbiota abundance with phylotypes *Actinobacteria* and *Synergistetes*, bacteria from classes *Actinomycetia, Coriobacteriia, Synergistia*, geni *Anaerobutyricum*, *Anaerostipes*,

Bifidobacterium, *Coprococcus*, *Dialister*, species *Bifidobacterium adolescentis, Bifidobacterium bifidum, Faecalibacterium prausnitzii, Anaerobutyricum hallii* and others is significantly higher than in patients with disease duration of less than 10 years. The data on the dominant and minor bacterial taxons in HC patients with various long-term prognoses have applied relevance, since they can underlie an idea of microbiota modulation and the use of microbiome-associated interventions.

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Малаева Е. Г. (ORCID ID: https://orcid.org/0000-0003-1051-0787): концепция и дизайн исследования, сбор, анализ и интерпретация данных, обоснование и написание рукописи

Стома И. О. (ORCID ID: https://orcid.org/0000-0003-0483-7329): концепция и дизайн исследования, проверка критически важного интеллектуального содержания, редактирование рукописи, окончательное утверждение рукописи для публикации

Author Contribution:

All the authors contributed significantly to the study and the article, read and approved the final version of the article before publication

Malaeva E. G. (ORCID ID: https://orcid.org/0000-0003-1051-0787): research concept and design, data collection, analysis and interpretation, justification and manuscript writing

Stoma I. O. (ORCID ID: https://orcid.org/0000-0003-0483-7329): concept and design of the study, verification of critical intellectual content, editing of the manuscript, final approval of the manuscript for publication

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