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Research Article

Gut Microbiota and Chronic Liver Disease in Children

Galina Vasilyevna Volynets[1]*, Alexander Sergeevich Potapov[2], Artyom Vyacheslavovich Nikitin[1,3], Vasilisa Valerievna Dudurich[4], Lavrenty Glebovich Danilov[5] and Tamara Andreevna Skvortsova[1,3]

[1]Veltischev Research and Clinical Institute for Pediatrics and Pediatric Surgery, Pirogov Russian National Research Medical University, Moscow, Russia

[2]Gastroenterology with the Hepatological Group, National Research Center for Children's Health of the Ministry of Health of Russia, Moscow, Russia

[3]Gastroenterological, Morozov Children's Municipal Clinical Hospital of the Moscow City Health Department, Moscow, Russia

[4]Head of the Microbiome, Laboratory "CERBALAB", St. Petersburg, Russia

[5]Genetics and Biotechnology, St. Petersburg State University, St. Petersburg, Russia

***Corresponding Author:** Galina Vasilyevna Volynets, Chief Researcher of the Department of Gastroenterology of the Veltischev Research and Clinical Institute for Pediatrics and Pediatric Surgery of the Pirogov Russian National Research Medical University, Professor Department of Innovative Pediatrics and Pediatric Surgery Faculty of Additional Professional Education Pirogov Russian National Research Medical University, Moscow, Russia.

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Abstract

Background: The study of the significance of the "liver-gut" axis and the influence of the intestinal microbiota on the formation of chronic liver diseases in children is extremely important.

Aim: To investigate the taxonomic diversity of the gut microbiota in children with chronic liver diseases compared with healthy patients, to identify differences in bacterial diversity in autoimmune and non-autoimmune liver diseases, as well as the impact of immunosuppressive therapy on the intestinal microbiota.

Methods: A metagenomic analysis of the gut microbiota of 24 children with chronic liver diseases (mean age 10.3 ± 4.7 years) was carried out with the identification of the V3-V4 region of the 16S rRNA gene. The group included 18 children with autoimmune liver diseases and 6 children with non-autoimmune liver diseases. The control group consisted of fecal samples of 34 apparently healthy children.

Results: When comparing fecal samples of children with autoimmune liver diseases with samples of healthy children, the taxa of *Bacteroides dorei, Collinsella aerofaciens, Ruminococcus caffidurs* prevailed, and for children of the control group - *Neisseria flavescens*.

When comparing samples of patients with non-autoimmune liver diseases and the control group, it was found that the taxa *Bacteroides fragilis, Klebsiella pneumoniae, Bifidobacterium longum* prevailed in healthy children.

When comparing fecal samples from children with autoimmune and non-autoimmune liver diseases, it was found that *Veillonella dispar, Cloacibacillus porcorum, Veillonella parvula, Prevotella histicola* and *Bacteroides eggerthii* taxa dominate in patients with non-autoimmune diseases. No dominant taxa of the gut microbiota were found in children with autoimmune liver diseases.

In patients receiving immunosuppressive therapy, the taxa *Veillonella dispar, Faecalibacterium prausnitzii, Roseburia inulinivorans, Bacteroides xylanisolvens*, and *Alistipes obesi* predominate, while in patients not receiving immunosuppressive therapy, the taxa *Phascolarctobacterium succinatutens, Bacteroides ovatus, Solobacterium moorei* and *Holdemanella massilien* predominate.

Conclusion: A study of the gut microbiota in children with chronic liver disease shows differences in gut microbiota imbalance compared to published findings in adults. The study of the gut microbiota can become an alternative to histological examination in the diagnosis and choice of tactics for the treatment of liver diseases. The gut microbiota model is capable of distinguishing autoimmune liver diseases from non-autoimmune diseases. Immunosuppressive therapy is accompanied by the dominance of taxa that reduce the production of short-chain fatty acids.

Keywords: *Gut Microbiota; Children; Chronic Liver Disease; Autoimmune Liver Disease; Non-Autoimmune Liver Disease; Immunosuppressive Therapy*

Core Tip

- The taxonomic composition of the intestinal microbiota in children with chronic liver disease is significantly different from that in healthy children.
- The diversity of the gut microbiota differs between children with autoimmune and non-autoimmune liver diseases.
- Immunosuppressive therapy affects the diversity of the gut microbiota.

Introduction

Autoimmune liver diseases include autoimmune hepatitis (AIH), autoimmune sclerosing cholangitis (ASC), and AIH de novo after liver transplantation [1]. AIH is a chronic immune-mediated liver disease characterized by hepatocyte destruction, circulating autoantibodies, and elevated serum IgG levels [1,2]. AIH occurs in both children and adults with a female predominance, and in recent years, the incidence of the disease tends to increase [3]. In the diagnosis of AIH, a liver biopsy is important, which facilitates the exclusion of alternative diseases, helps assess the degree of inflammation and the severity of liver fibrosis, as well as make therapeutic decisions [1,4]. In general, AIH responds favorably to standard immunosuppressive therapy, while a minority of patients who do not respond to standard treatment may rapidly develop fibrosis and cirrhosis [5,6]. The etiology of AIH is unknown, although both genetic and environmental factors are involved in its development. Loss of tolerance to liver antigens induced by environmental agents such as xenobiotics and pathogens in genetically susceptible individuals is believed to initiate the disease [3,6]. Serious efforts have been made to determine the genetic architecture of AIH, but it has been reproducibly confirmed that only risk loci in the major histocompatibility complex (HLA) have been identified as predisposing to the disease [7,8].

Evidence is accumulating that the gut microbiota, containing many more genes than the human genome, has become a key environmental factor involved in the development of liver diseases along the liver-gut axis [9-13]. Preliminary evidence for the involvement of the gut microbiota in the pathogenesis of AIH is presented in a mouse model [14,15]. A correlation has been found between three genera of the oral microbiota and salivary inflammatory cytokines with AIH [16,17].

At the taxonomic level, the structure of the diverse and dynamic microbial community of the gut microbiota varies greatly between individuals and populations [18], although its biochemical functions at baseline are usually stable [19]. However, the stability of the structure and function of this community may vary depending on dietary changes [20,21], antibiotic use [22] and exposure to xenobiotics [23]. Immunosuppressive therapy leads to persistent changes in the intestinal microbiota with a constant increase in the number of

proteobacteria, including opportunistic pathogens [24]. And the members of this community can disappear from the microbiota, which leads to the loss of their species (and biochemical) diversity [25].

Most of the studies on the effect of drugs on the intestinal microbiota have been conducted in adults, are descriptive in nature and require further in-depth study.

Aims of Research

The purpose of this research is to study the differences in the taxonomic diversity of fecal microbiota in children with chronic liver diseases compared with healthy children with the isolation of the V3-V4 region of the *16S rRNA* gene, to establish the existing differences in the gut microbiota in autoimmune and non-autoimmune liver diseases, and to evaluate the differences depending on the implementation of immunosuppressive therapy.

Patients and Methods

A metagenomic analysis of the gut microbiota of 24 children with chronic liver diseases aged 2 to 17 years (mean age 10.3 ± 4.7 years) was carried out, with the identification of the V3-V4 region of the *16S rRNA* gene. The group included 18 patients with autoimmune liver diseases, among which there were 13 children with AIH (of which 5 children were diagnosed with liver cirrhosis in the outcome of AIH), 2 cases of overlap syndrome (AIH + ASC), 3 patients with ASC. The diagnosis of autoimmune liver diseases was established in accordance with the ESPGHAN guidelines [1]. The comparison group consisted of 6 children with non-autoimmune liver diseases: one patient with type 1 hereditary tyrosinemia with liver cirrhosis, one patient with Alagille syndrome, one with biliary tract hypoplasia, three with hepatic form of Wilson's disease.

The study is continuous - the material (feces) was collected simultaneously from all children with liver diseases who were under examination at the time of the collection of the material.

The control group consisted of fecal samples from 34 apparently healthy children who were age-matched to patients with autoimmune liver diseases, had normal liver function tests, normal fasting blood glucose, blood lipids, no hepatitis B and/or C virus antigen, and were not taking antibiotics within 4 weeks prior to sample collection.

Of the examined 24 children, 12 patients with autoimmune liver diseases received immunosuppressive therapy (glucocorticosteroids or glucocorticosteroids in combination with azathioprine).

The study protocols were approved by independent local ethical committees and academic councils of the Federal State Autonomous Institution "National Medical Research Center for Children's Health" of the Ministry of Health of the Russian Federation and the State Budgetary Institution of Health of the City of Moscow "Morozov Children's City Clinical Hospital of the Department of Health of the City of Moscow", which observed the patients.

The metagenomic study of fecal samples was carried out in the genetic laboratory of the Medical Genetic Center CERBALAB (St. Petersburg).

Bioinformatic analysis of 16S *rRNA* sequencing

The study was conducted at Saint Petersburg State University. The 16S rRNA sequencing data was analyzed using a bioinformatics pipeline implemented in the R v.3.6 (R Core Team, 2014) and Python programming languages. In the first stage of the pipeline, primer sequences were truncated at the beginning of paired reads, while pairs of reads that did not contain primer sequences were discarded. Next, we trimmed 25 base pairs from the end of each read as low-quality bases and processed the resulting data using the DADA2 pipeline to identify exact sequence variants [26]. After exact sequence variants were identified, the forward and backward reads were combined by concatenation, and the resulting sequences were used for naive Bayesian taxonomic classification [27] using the SILVA v138 database as

a reference [28]. Microbial species identification was performed using the exact match algorithm in DADA2 using SILVA v138 sequences, pre-processed accordingly using custom scripts.

Statistical processing

Comparison of the abundance of different taxa in different cohorts was carried out using the Mann–Whitney U test (for paired comparisons). Multiple tests were corrected using the Benjamin-Hochberg method in R. To calculate the Shannon Diversity Index, a matrix containing the total number of ASVs at the species level per specimen was provided as input to the "vegan" package in the R programming language. To identify special taxa for each group, sPLS-DA analysis was carried out using the "muliomix" package in the R programming language.

Results

The conducted study revealed 684 types of microorganisms in the studied samples of patients' faeces.

The analysis of the conducted studies showed that fecal samples of healthy children and patients with autoimmune liver diseases have differences in the taxonomic diversity of the gut microbiota. For healthy patients and patients with autoimmune liver diseases, the Shannon diversity index differs. The Wilcoxon test showed differences between the groups (W = 443, p-value = 0.001355). For the group of patients with autoimmune diseases, sPLS-DA analysis showed the predominance of taxa: *Bacteroides dorei, Collinsella aerofaciens, Ruminococcus caffidurs*, and for children in the control group - *Neisseria flavescens* (Figure 1).

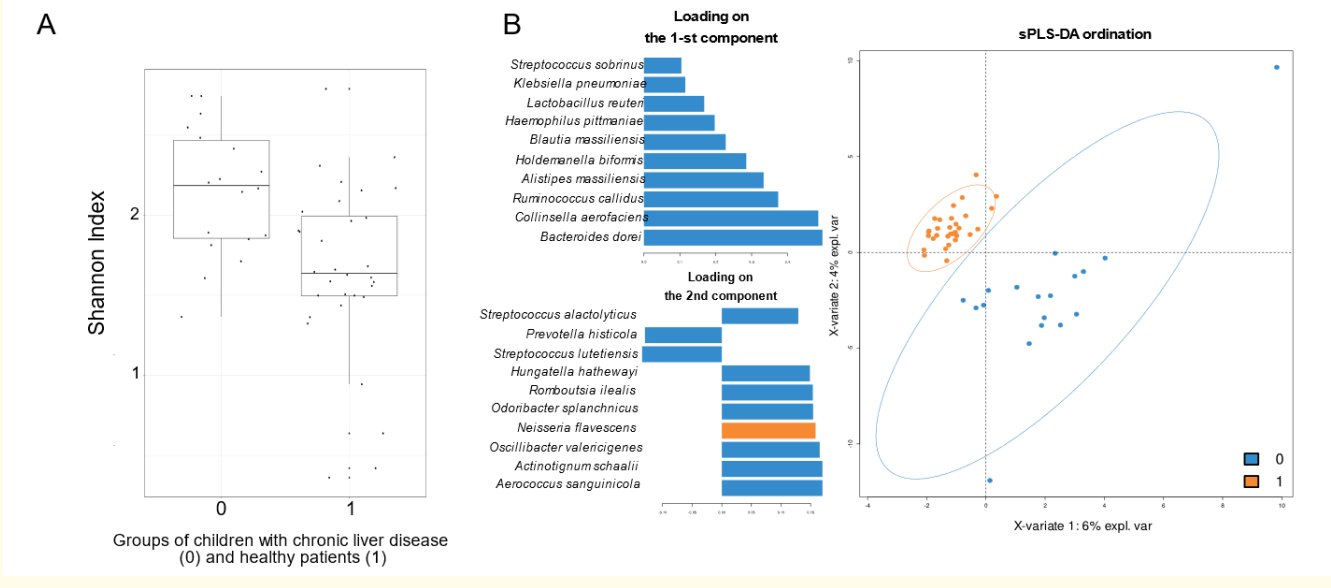

Figure 1: *Taxonomic diversity of the gut microbiota in healthy children and children with autoimmune liver disease. Samples of children with autoimmune liver diseases (0) and samples of healthy children (1) have differences in taxonomic diversity. A. The Shannon Diversity Index is different for healthy patients and patients with autoimmune liver disease. The comparison was carried out using the Wilcoxon test and showed differences between the groups (W=443, p-value=0.001355). B. For the group of patients with autoimmune liver diseases (0) sPLS-DA analysis showed the predominance of taxa: Bacteroides dorei, Collinsella aerofaciens, Ruminococcus caffidurs, and for the group of healthy children (1 - control group) - Neisseria flavescens.*

When comparing samples of patients with non-autoimmune diseases and samples of healthy patients, it was found that the Shannon diversity index in the compared groups of patients has no significant differences. Comparison with the Wilcoxon test showed a difference between the groups (W = 149, p-value = 0.03303). sPLS-DA analysis showed that in healthy children there is a predominance of taxa: *Bacteroides fragilis, Klebsiella pneumoniae, Bifidobacterium longum* (Figure 2).

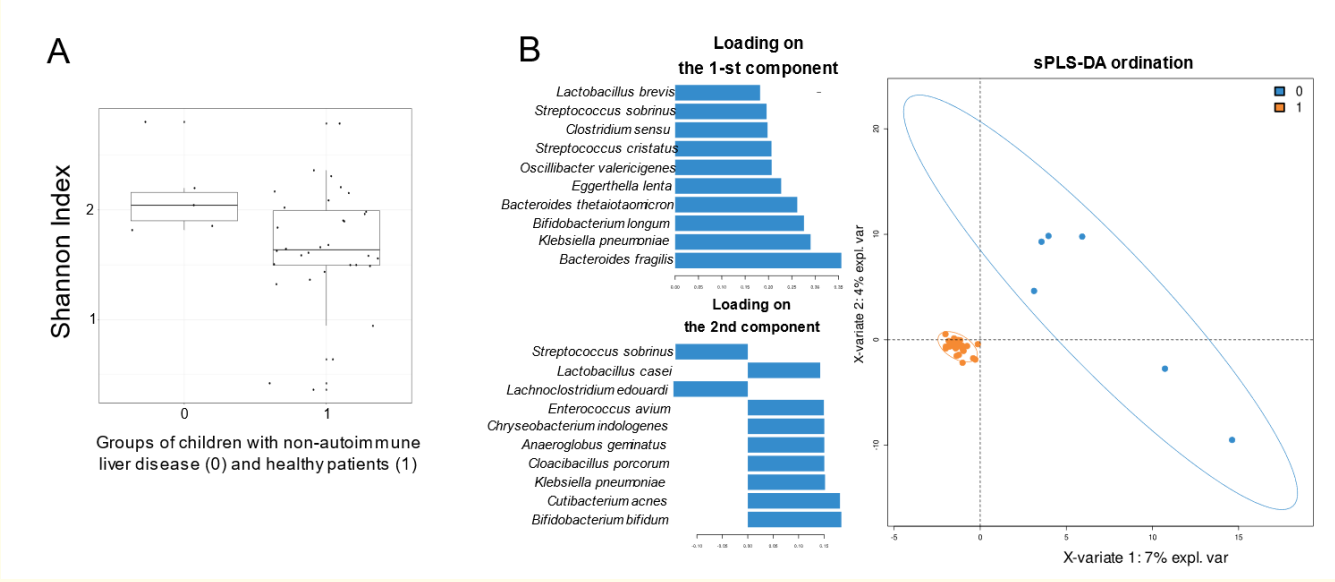

Figure 2: *Taxonomic diversity of the gut microbiota in healthy children and children with non-autoimmune liver diseases. Samples from children with non-autoimmune liver diseases and samples from healthy patients. A. The Shannon Diversity Index does not differ between patients with non-autoimmune liver disease (0) and healthy patients (1). Comparison using the Wilcoxon test showed no difference between the groups (W=149, p-value=0.03303). B. For a group of healthy patients, sPLS-DA analysis showed the predominance of taxa: Bacteroides fragilis, Klebsiella pneumoniae, Bifidobacterium longum.*

A comparative analysis of the distribution of taxa of microorganisms in the intestinal microbiota for patients with autoimmune and non-autoimmune liver diseases showed that there were no differences in taxonomic diversity (Figure 3). The Shannon Diversity Index does not differ between patients with autoimmune liver disease and patients with non-autoimmune liver disease. Comparison using the Wilcoxon test showed no difference between the groups (W = 50, p-value = 0.8204). However, the use of the sPLS-DA method, which makes it possible to identify taxa characteristic of each group when constructing ordination, showed that such taxa as *Veillonella dispar, Cloacibacillus porcorum, Veillonella parvula, Prevotella histicola* and *Bacteroides eggerthii* predominate in patients with non-autoimmune liver diseases (Figure 4). No dominant taxa of the gut microbiota were found in children with autoimmune liver diseases.

A comparative analysis of the distribution of taxa of microorganisms of the gut microbiota in patients who receive and do not receive immunosuppressive therapy showed that the Shannon diversity index does not differ for these groups of patients. Comparison using the Wilcoxon test showed no difference between the groups (W = 71, p-value = 0.9774) (Figure 5). However, sPLS-DA analysis showed that in patients receiving immunosuppressive therapy, such taxa as *Veillonella dispar, Faecalibacterium prausnitzii, Roseburia inulinivorans, Bacteroides xylanisolvens* and *Alistipes obesi* dominate. While in patients not receiving immunosuppressive therapy, *Phascolarctobacterium succinatutens, Bacteroides ovatus, Solobacterium moorei, Holdemanella biformis* and *Blautia massiliensis* dominate (Figure 6).

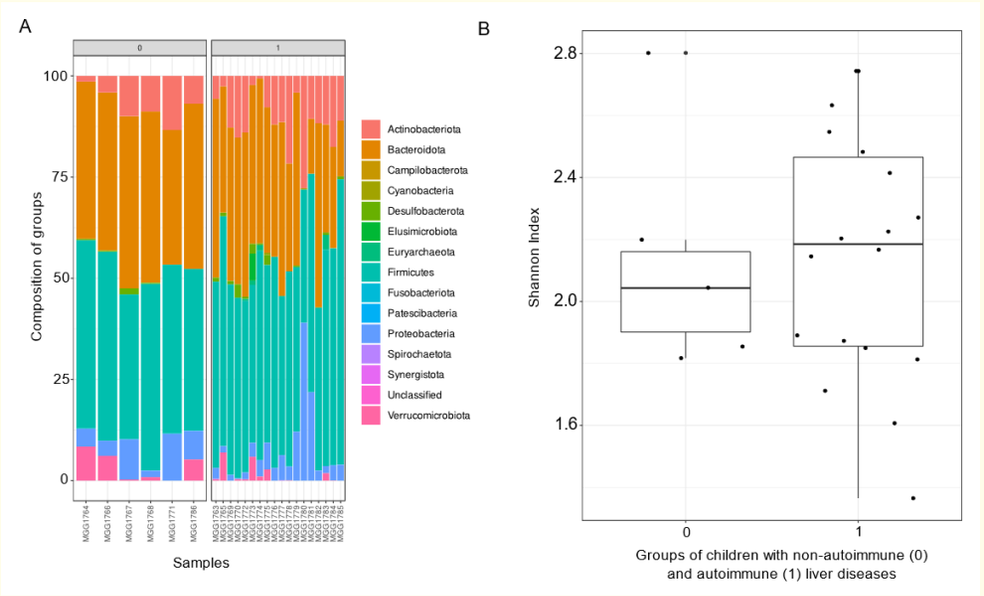

Figure 3: *Taxonomic diversity of the gut microbiota in children with non-autoimmune and autoimmune liver diseases. Patient samples with non-autoimmune and autoimmune liver diseases do not differ in taxonomic diversity. A. Distribution of taxa of microorganisms of the intestinal microbiota for patients with non-autoimmune liver diseases (0) and patients with autoimmune liver diseases (1). B. The Shannon Diversity Index does not differ between patients with autoimmune liver disease and patients with non-autoimmune liver disease. Comparison using the Wilcoxon test showed no difference between the groups (W=50, p-value=0.8204).*

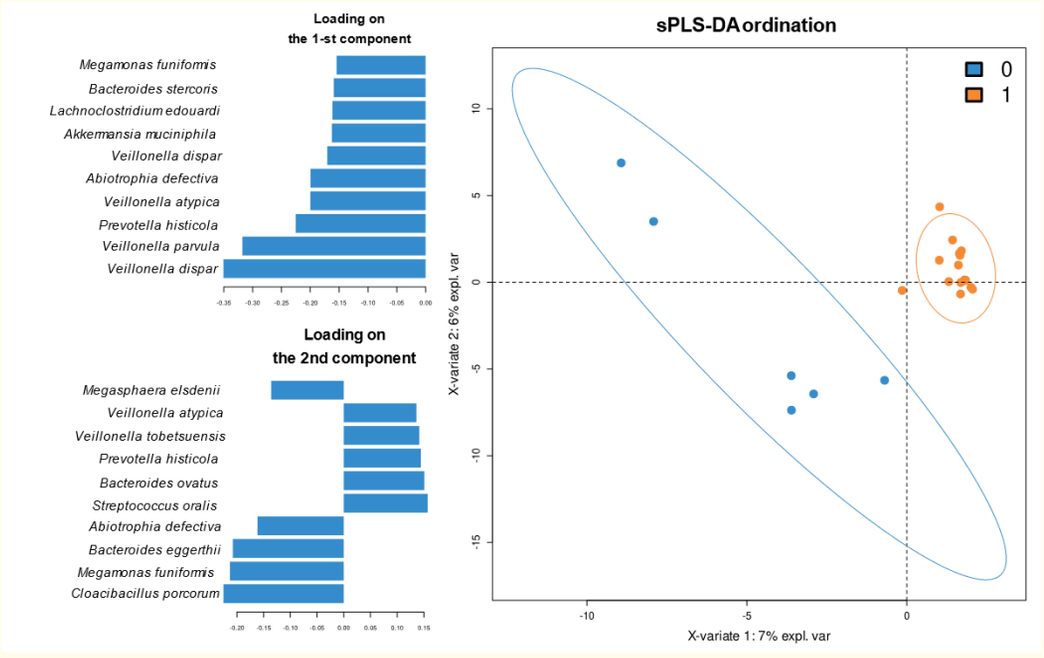

Figure 4: *Taxonomic composition of the gut microbiota in children with non-autoimmune (0) and autoimmune (1) liver diseases. Analysis of sPLS-DA showed that the following taxa predominate in patients with non-autoimmune liver diseases: Veillonella dispar, Cloacibacillus porcorum, Veillonella parvula, Prevotella histicola and Bacteroides eggerthii.*

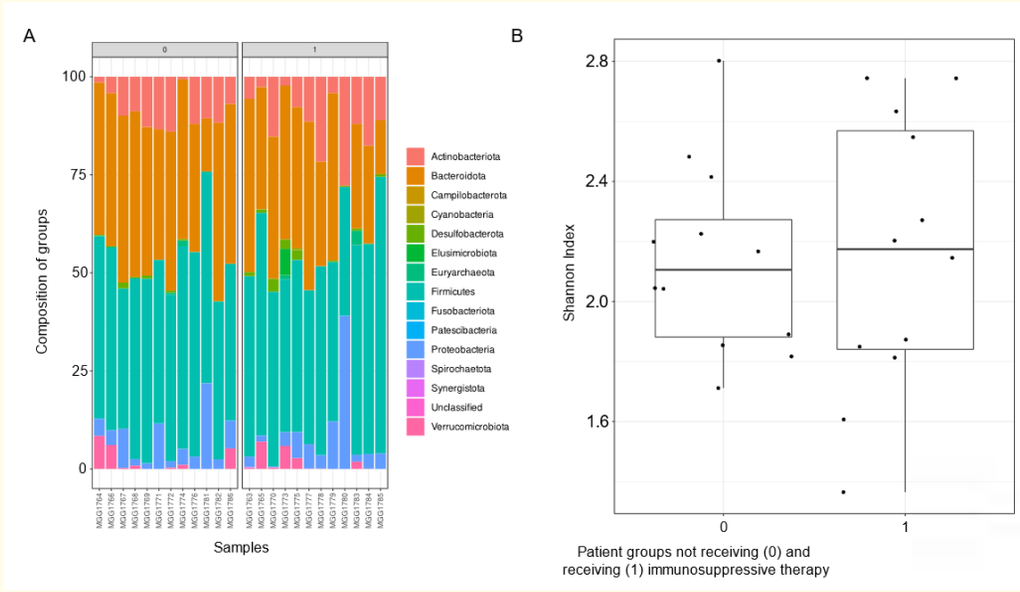

Figure 5: *Taxonomic composition of the gut microbiota in children with chronic liver disease who receive immunosuppressive therapy compared with patients who do not receive immunosuppressive therapy. Samples from patients receiving immunosuppressive therapy do not differ in the taxonomic diversity of the gut microbiota from those of patients not receiving immunosuppressive therapy. A. Distribution of taxa for patients receiving immunosuppressive therapy and patients not receiving immunosuppressive therapy. B. The Shannon Diversity Index does not differ between patients not receiving immunosuppressive therapy (0) and patients receiving immunosuppressive therapy (1). Comparison was performed using the Wilcoxon test and showed no difference between the groups (W=71, p-value=0.9774).*

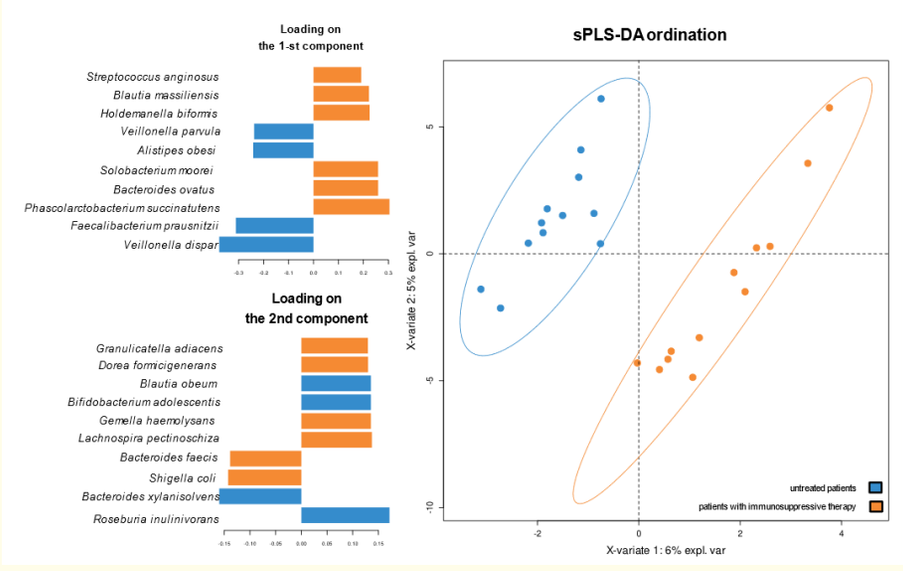

Figure 6: *Differences in the taxonomic diversity of the gut microbiota in samples from patients who receive immunosuppressive therapy and patients who do not receive immunosuppressive therapy. Analysis of sPLS-DA showed that Veillonella dispar, Faecalibacterium prausnitzii, Roseburia inulinivorans, Bacteroides xylanisolvens, and Alistipes obesi taxa dominate in patients not receiving immunosuppressive therapy. In patients receiving immunosuppressive therapy, the following taxa dominate: Phascolarctobacterium succinatutens, Bacteroides ovatus, Solobacterium moorei, Holdemanella biformis, and Blautia massiliensis.*

Discussion

There is growing evidence that changes in the gut microbiome are correlated with almost all known liver or immunological diseases [9,13,29,30]. In our study, using *16S rRNA* gene sequencing, we described the structure of the gut microbiota community in children with chronic liver disease.

The imbalance of the gut microbiota in AIH in adults was characterized by an increased relative content of *Veillonella, Streptococcus, Klebsiella* and *Lactobacillus*, as well as a decrease in the content of many bacteria. It was also shown that the genera of microorganisms that proliferate in AIH were increased in the fecal microbiota in primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [10,12], which served as the basis for combining children with AIH and autoimmune cholangitis into one group in our study. A decrease in the number of obligate anaerobes and an increase in the number of facultative anaerobes, including *Streptococcus, Klebsiella*, and *Lactobacillus*, were observed in the faeces of adult patients with AIH, indicating that the microbial community has shifted towards more aerotolerant microbes [27]. In our study, in children with autoimmune liver diseases, the predominance of the taxa *Bacteroides dorei, Collinsella aerofaciens, Ruminococcus caffidurs* was shown, while in healthy patients from the control group, the taxon *Neisseria flavescens* prevailed - a bacterium that actively reduces oxygen, which reduces the redox potential of the environment habitat and creates conditions for the development of anaerobic microflora.

Bacteroides dorei have a direct effect on the metabolism of microorganisms that produce lipopolysaccharides (LPS) in the human intestine. They reduce the production of LPS by the intestinal microbiota, which contribute to the strength of the intestinal barrier. Gut microbiota-derived LPS and systemic endotoxemia are involved in the onset and progression of common diseases such as inflammatory bowel disease (IBD), obesity and related metabolic diseases, and non-alcoholic steatohepatitis [28-30]. It has been shown that in the intestinal microbiome of adults with AIH, the biosynthesis of LPS, which induces pro-inflammatory cytokines, is altered, as well as amino acid metabolism, which can be regulated by intestinal bacteria with the formation of immunomodulatory metabolites [31]. This suggested that treatment with *Bacteroides* could serve as a new and attractive therapeutic strategy to suppress the inflammatory response in these diseases [32].

Collinsella aerofaciens and *Ruminococcus caffidurs* are microorganisms that produce short chain fatty acids (SCFA) by fermenting dietary fiber [33,34]. SCFAs are known to have beneficial effects on health through their anti-inflammatory effects. It has been shown that a decrease in SCFA production by the microbiota increases the oxygen concentration in the intestinal lumen in mice, which leads to the spread of facultative anaerobes [35]. A decrease in the number of obligate anaerobes and an increase in the number of facultative anaerobes in adults [27] and the replacement of anaerobes by facultative anaerobes can be a common manifestation in various disease states, including chronic liver diseases [34]. Our study indicates that the gut microbiota imbalance in children with AIH is significantly different from that in adults, which necessitates multicenter studies to develop further approaches to correcting the intestinal microbiota in children with chronic liver diseases.

As for the effect of immunosuppressive therapy on the gut microbiota in children, quite interesting results have also been obtained here. Children with autoimmune liver disease received corticosteroids or a combination of corticosteroids with azathioprine, drugs that remain a major part of the treatment strategy for these diseases. In adult studies, changes in the gut microbiota induced by corticosteroid treatment in a mouse model have been shown to reduce bacterial richness and diversity [36] and alter the global composition of the gut microbiota [37]. At the level of types of microorganisms, the literature data are heterogeneous. An increased *Firmicutes/Bacteroidetes* ratio has been shown [37], while other studies have found a decrease in *Firmicutes* [36], *Bacteroidetes, Actinobacteria*, alpha- and gamma-proteobacteria [36], and *Deferribacteres* [38]. Glucocorticosteroids have been reported to increase the relative abundance of fecal *Clostridiales, Lactobacillus* [36], *Anaerostipes* [38], *Bifidobacterium* [39] and decrease *Oscillospira, Bilophila* and *Rikenella* [38]. In addition, two studies have found a reduction in *Mucispirillum* [36,40], a mucin-degrading bacteria [41] that is involved in T-cell maturation and activation through interaction with antigen-presenting cells [42]. Physiologically, the gut microbiota is capable of resilience, defined as its ability to return to its original state after being disturbed [43]. Administration of dexamethasone to mice has been shown to increase the

delay in the development of gut microbiota resistance after severe *Clostridium difficile* infection [44]. Finally, glucocorticosteroids reduce the abundance of *Clostridium sensu stricto* in the ileum [37].

Azathioprine inhibits the proliferation of several intestinal bacteria *in vitro*: *Campylobacter concisus, Bacteroides fragilis* and *Bacteroides vulgatus* [45]. *E. coli* growth was inhibited only by the highest concentration of azathioprine (200 μg/ml). Azathioprine had no significant effect on the growth of *E. faecalis*. In a cohort of 20 patients with IBD, azathioprine was found to increase the concentration of mucosal bacteria compared to healthy controls and the percentage of epithelial surface covered with attached bacteria compared to patients with IBD [46].

In our study, in children receiving immunosuppressive therapy, such taxa of gut microbiota as *Veillonella dispar, Faecalibacterium prausnitzii, Roseburia inulinivorans, Bacteroides xylanisolvens*, and *Alistipes obesi* dominate. While in patients not receiving immunosuppressive therapy, *Phascolarctobacterium succinatutens, Bacteroides ovatus, Solobacterium moorei, Holdemanella biformis* and *Blautia massiliensis* dominate.

Of note, a study of the gut microbiota in steroid-naïve adults with AIH showed lower alpha diversity. The depletion of obligate anaerobes and the expansion of potential pathobionts, including *Veillonella*, has been associated with disease status. Most strongly associated with the disease were *Veillonella dispar*, which positively correlated with serum aspartate aminotransferase levels and liver inflammation, and the combination of *Veillonella, Lactobacillus, Oscillospira*, and *Clostridiales* had a potentially high diagnostic value for individuals with AIH [27]. *Veillonella dispar* were among the most dominant in adult PSC patients [47]. In our study of the gut microbiome in children receiving immunosuppressive therapy for autoimmune liver diseases, one of the most dominant was the taxon *Veillonella dispar*.

Microorganisms such as *Phascolarctobacterium succinatutens*, which dominate in our study in children with autoimmune liver diseases who were not receiving immunosuppressive therapy at the time of the study, can utilize succinic acid produced by other intestinal bacteria, converting it into propionic acid by decarboxylation [48]. Adult studies have shown that *Phascolarctobacterium* decreases in IBD, which can lead to accumulation of succinic acid and, accordingly, to a decrease in propionate levels [49].

Thus, in the absence of immunosuppressive therapy, an increase in the number of *Phascolarctobacterium succinatutens* may contribute to an increase in SCFA [49]. In patients who do not receive immunosuppressive therapy, *Bacteroides ovatus* also dominates, which forms acetic, propionic, isobutyric and isovaleric acids, and also selectively affects the presence of neurotransmitters in the intestine [50].

In the present study, a gut microbiota model consisting of *Bacteroides dorei, Collinsella aerofaciens, Ruminococcus caffidurs* is able to distinguish autoimmune liver diseases from non-autoimmune diseases characterized by a gut microbiota model dominated by *Veillonella dispar, Cloacibacillus porcorum, Veillonella parvula, Prevotella histicola*, and *Bacteroides eggerthii*.

Our study also showed that immunosuppressive therapy in children with chronic liver disease is accompanied by the dominance of the taxa *Veillonella dispar, Faecalibacterium prausnitzii, Roseburia inulinivorans, Bacteroides xylanisolvens* and *Alistipes obesi* in the gut microbiota, which may be accompanied by a decrease in the formation of SCFA, while in patients not those receiving immunosuppressive therapy are dominated by: *Phascolarctobacterium succinatutens, Bacteroides ovatus, Solobacterium moorei, Holdemanella biformis* and *Blautia massiliensis*, which contributes to the formation of SCFA.

Conclusion

The main advantages of our study are that it was conducted in children with chronic liver diseases, and the results show differences in the imbalance of the intestinal microbiome in these diseases compared with the results obtained in adults. Factors such as medication, diet, and environmental factors may have influenced the results of our study. The confounding effect of various drugs, which may confound their effect on gut microbial composition, has probably not been carefully assessed. In addition, the current study did not take into account other drugs that were prescribed to patients. The small number of centers participating in the study may limit the application of

the microbiota-based diagnostic model. Multicenter studies involving subjects from different regions will be required to generalize these results. Finally, this study provides evidence for an association rather than a causal relationship. Further research is needed to evaluate the role of disease-associated bacteria in immune dysfunction and liver inflammation.

The study of the intestinal microbiota can become an alternative to histological examination in the diagnosis and choice of tactics for the treatment of liver diseases. There is an unmet need to find non-invasive biomarkers to assess liver inflammation and fibrosis in AIH.

Given the small cohort used, the work carried out may not have sufficient statistical power to detect changes in the microbial profile. However, the results of our study help to establish the imbalance of the intestinal microbiota in chronic liver diseases and use it in the differential diagnosis of these diseases.

Author Contributions

Volynets GV, Potapov AS designed and coordinated the study.

Nikitin FV, Dudurich VV, Danilov LG, Skvortsova TA performed the experiments, acquired and analyzed data.

Volynets GV, Potapov AS, Dudurich VV, Danilov LG interpreted the data.

Volynets GV, Potapov AS wrote the manuscript.

All authors approved the final version of the article.

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