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Intestinal microbiota in obese children with non-alcoholic fatty liver disease depending on the gallbladder function

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Aim. To determine the features of the intestinal microbiota in obese children with non-alcoholic fatty liver disease (NAFLD) depending on the functional state of the gallbladder.

Materials and methods. A total of 73 children aged 10–17 years (mean age – 12.15 ± 2.51 years) were examined. According to body mass index, transient elastography (Fibroscan®), and ultrasound data, the patients were divided into 3 groups: group I – 35 obese children with NAFLD and gallbladder hypokinesia, group II – 30 obese children with NAFLD and gallbladder normokinesia, group III (control) – 8 healthy children with normal weight and gallbladder normokinesia. Contractile function of the gallbladder was assessed by ultrasound examination after physiological food loading. Small intestinal bacterial overgrowth (SIBO) and lactose absorption were assessed with the hydrogen breath test (HBT) with lactose loading using a Gastrolyzer (Bedfont Scientific Ltd, UK). Qualitative and quantitative intestinal microbiome composition were studied using bacterial culture method with ten-fold dilutions (10^{-1}–10^{-9}) on standard sets of selective and differential-diagnostic culture media for the isolation of aerobic and anaerobic microbes. Fecal short-chain fatty acid (SCFA) content was evaluated with gas chromatography (Chromatec-Crystal-5000).

Results. Lactose-dependent SIBO was observed in almost half of NAFLD patients (42.9 %) without significant differences depending on functional activity of the gallbladder. Patients with decompensated dysbiosis predominated in group I children (37.1 % of patients). In group II children, the subcompensated dysbiosis was more common (36.7 % of patients). The concentration of *Lactobacillus* and *Enterococcus* in group I patients was significantly 1.9 times ($P < 0.05$) and 1.4 times ($P < 0.05$) lower, respectively, than that in group II patients. The level of fecal acetic acid and butyric acid in group I children was 6.9 and 2.0 times ($P < 0.05$) increased, respectively, compared to control group, assuming bile acids involvement in the regulation of microbiome composition.

Conclusions. Impaired contractile function of the gallbladder in NAFLD children is associated with a sharp decrease in the number of major symbionts of the intestinal microbiota as well as increased production of acetic and butyric SCFA.

Key words: gallbladder, hypokinesia, non-alcoholic fatty liver disease, microbiota, short-chain fatty acids.

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Мікробіота кишківника в дітей з ожирінням і неалкогольною жировою хворобою печінки залежно від функції жовчного міхура

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Мета роботи – визначити особливості мікробіоценозу тонкого та товстого кишечника в дітей із неалкогольною жировою хворобою печінки (НАЖХП) залежно від функціонального стану жовчного міхура (ЖМ).

Матеріали та методи. Обстежили 73 дітей із НАЖХП віком від 10 до 17 років. Відповідно до індексу маси тіла, даних транзієнтної еластографії (Fibroscan®) та ультразвукового дослідження пацієнтів поділили на групи: I – 35 дітей із НАЖХП, ожирінням та гіпокінезією ЖМ; II – 30 дітей із НАЖХП, ожирінням та нормокінезією ЖМ; III (контрольна) – 8 відносно здорових дітей із нормальною масою тіла та нормокінезією ЖМ. Моторно-евакуаторну функцію ЖМ оцінювали з використанням фізіологічного харчового навантаження. Хроматографію коротколанцюгових жирних кислот у копрофільтраті пацієнтів здійснили на хроматографі Хроматек-Кристал-5000. Видовий і кількісний склад мікрофлори вмісту товстої кишки визначили методом посіву десятикратних розведень (10^{-1}–10^{-9}), використавши стандартний набір елективних і диференційно-діагностичних поживних середовищ для виділення аеробних та анаеробних мікроорганізмів. Діагностику синдрому надлишкового бактеріального росту виконали за допомогою водневого дихального тесту з навантаженням із лактозою, використали газоаналізатор «Gastro» Gastrolyzer (Bedfont Scientific Ltd, Велика Британія).

Результати. Лактозозалежний синдром надлишкового бактеріального росту визначили майже у половини хворих (42,9 %) на НАЖХП незалежно від функціональних змін ЖМ. У дітей із НАЖХП і гіпофункцією ЖМ частіше виявляли декомпенсовану форму дисбіозу (37,1 % випадків), з нормокінезією ЖМ – субкомпенсовану (36,7% випадків). Концентрація Lactobacillus (в 1,9 раза, $p < 0,05$) та Enterococcus (в 1,4 раза, $p < 0,05$) вірогідно нижча в пацієнтів з НАЖХП і гіпофункцією ЖМ, ніж у хворих із нормальною моторно-евакуаторною функцією ЖМ. Рівень фекальної оцтової кислоти в дітей із НАЖХП і гіпокінезією ЖМ збільшувався в 6,9 раза ($p < 0,05$), а рівень масляної кислоти – вдвічі ($p < 0,05$) порівняно з групою контролю. Це свідчить про участь жовчних кислот у регуляції якісного та кількісного складу кишкового мікробіому.

Висновки. Зниження скоротливої функції жовчного міхура в дітей із НАЖХП асоційоване з різким зменшенням кількості основних симбіонтів кишкового мікробіоценозу, збільшенням концентрації умовно-патогенної мікрофлори, а також підвищенням продукції оцтової та масляної коротколанцюгових жирних кислот.

Ключові слова: гіпокінезія, жовчний міхур, неалкогольна жирова хвороба печінки, мікробіота, коротколанцюгові жирні кислоти.

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The number of children with non-alcoholic fatty liver disease (NAFLD) has increased in recent years. The development of NAFLD in children is associated with obesity in 23–53 % of cases [1,2].

Fatty infiltration of the liver, pancreas, and biliary tract associated with digestive dysfunction is accompanied by the development of intestinal dysbiosis. Changes in intestinal microbiota, in turn, can promote systemic inflammation and contribute to the NAFLD progression [3,4]. Moreover, some evidence has been obtained that the accumulation of fat in the gallbladder (GB) wall in obese patients can lead to GB contractility impairment [5,6] which can also contribute to the NAFLD development and progression due to disruption of bile acid circulation [7].

Recent studies have shown that the intestinal microbiota plays an active role in the NAFLD pathogenesis through the control of bile acids circulation and bile acid profile modification [8]. Since bile acids are not only dietary lipids emulsifiers but also act as signaling molecules through binding to the G-protein-coupled bile acid receptor and farnesoid X receptors expressed both in the liver and in the intestine, they are involved in important metabolic processes such as glucose and lipid homeostasis and have a beneficial impact on host metabolism. Although intestinal bacteria control the production of secondary bile acids, bile acids themselves, in turn, can affect the gut microbiota composition, so may contribute to bacterial overgrowth development. It is currently not completely clear to what extent alterations in the intestinal microbiota of patients contribute to bile acid dysregulation, or whether bile acids affect the function and composition of the intestinal microbiome in NAFLD children.

An increase in the absolute number of microorganisms or changes in the qualitative composition may lead to increased intestinal permeability, bacterial translocation, activation of inflammatory pathways and progression of liver structural changes [9,10]. There is certain evidence that the intestinal barrier dysfunction as well as intestinal microbiota dysbiosis correlate with the severity of liver steatosis and fibrosis [8]. Furthermore, changes in intestinal microbiota may cause increased production and absorption of short-chain fatty acids (SCFA) from the intestine because some SCFA are produced by different species of colonic saccharolytic microflora through microbial digestion [11]. The SCFA ratio reflects the domination of certain bacterial species in the intestinal microflora [12].

Thus, the relationship between intestinal microbiota and the hepatobiliary system functionality in NAFLD remains a relevant target of pediatric research and requires further study.

Aim

The aim of the study: to determine intestinal microbiota features in obese children with NAFLD depending on the functional activity of the gallbladder.

Materials and methods

A total of 73 children aged 10–17 years (mean age – 12.15 ± 2.51 years) were enrolled. The materials provided for publication do not conflict with the bioethics. All patients and their parents give the written informed consent to conduct the study.

The inclusion criterion for the study was the presence of NAFLD and obesity. The exclusion criterions from the study were acute infections or other inflammatory diseases, chronic viral, autoimmune and toxic hepatitis, cholelithiasis.

The presence of NAFLD was proved by transient elastography (FibroScan®502 touch, Echosens, France) with controlled attenuation parameter (CAP) measurement. The contractile function of the GB was assessed with ultrasound using a physiological food loading: bread (40 g), butter (20–25 g), cheese 45–50 % (20 g), tea (150–200 ml) with sugar (5 g). The 34–64 % decrease in the GB volume by the 60th min corresponded to the normokinesia of GB, less than 34 % – hypokinesia.

According to body mass index, transient elastography, and ultrasound findings, the patients were divided into 3 groups: group I – 35 obese NAFLD children with GB hypokinesia, group II – 30 obese NAFLD children with GB normokinesia, group III (control) – 8 healthy children with normal weight and GB normokinesia.

Evaluation of fecal SCFA was performed via gas chromatography on a Crystal-5000 chromatograph using the Guohua Zhao method [12].

Determination of the species and quantitative composition of the colonic microflora were carried out by sowing tenfold serial dilutions (10^{-1}–10^{-9}) on standard sets of elective and differential-diagnostic nutrient media for the extraction of aerobic and anaerobic microorganisms. The number of colonies was counted to determine the population level of microorganisms. The grade of dysbiosis was assessed using step gradation, which indicated the amplitude of colonic microbiota deviations: absence of deviations – eubiosis; mild deviations – I degree dysbiosis (compensated); moderate deviations – II degree dysbiosis (subcompensated); severe deviations – III degree dysbiosis (decompensated) [13].

Small intestinal bacterial overgrowth (SIBO) was confirmed using a hydrogen breath test with lactose load using a gas analyzer "Gastro" Gastrolyzer (Bedfont Scientific Ltd, Great Britain). An increase in hydrogen content more than 10 ppm from the basal level during the first 30–60 minutes indicated the presence of lactose-dependent SIBO in the small intestine. An increase in hydrogen content of more than 10 ppm, as compared to the baseline level, at 120–180 min of the test was diagnosed as malabsorption of lactose (ML).

Statistical analysis of the results was performed using the software package Statistica 6.1 (serial number AGAR909 E415822FA). The comparison of the mean values of the variables was carried out by the parametric method (Student's t-criterion) for the distribution of these characteristics. The variation of the distribution type was additionally checked for normality by the Shapiro–Wilk's test. The non-parametric method (U-criteria of the Mann–Whitney test) was used in the other cases. The difference in indicators was considered significant at a level of $P < 0.05$. Correlation analysis was performed according to the Spearman rank-order correlation coefficient.

Results

The frequency of lactose-dependent SIBO detection in NAFLD children was 42.9 %, it was higher in children with GB hypokinesia (45.7 %) compared to children with

Table 1. Concentration of hydrogen (ppm) in the examined children depending on the GB function, M ± m

Measurement time	Control group (n = 8)	Group I (n = 35)	Group II (n = 30)
0 min (I)	4.63 ± 1.78	6.63 ± 0.64	7.27 ± 2.09
15 min (II)	6.63 ± 1.53	9.23 ± 0.90	8.33 ± 1.85
30 min (III)	6.13 ± 1.53	14.97 ± 2.31*	11.67 ± 2.30
45 min (IV)	8.50 ± 2.94	40.94 ± 3.91	44.93 ± 2.92
60 min (V)	10.13 ± 5.03	46.37 ± 3.48*	45.97 ± 3.93*
90 min (VI)	21.38 ± 15.83	59.71 ± 3.36	47.57 ± 3.97*
120 min (VII)	26.00 ± 21.73	39.29 ± 3.19	38.67 ± 3.87
150 min (VIII)	13.63 ± 18.79	26.11 ± 2.84	25.47 ± 3.51
180 min (IX)	10.75 ± 11.68	21.51 ± 2.22	20.80 ± 2.17
Average indicator	16.63 ± 3.00	24.91 ± 2.59	23.61 ± 2.61

*: P < 0.05 – significance of the differences according to t-criterion in comparison with the control group.

Table 2. The spectrum of fecal SCFA in children depending on the GB function, M ± m

Biochemical indicator, μg/ml	Control group (n = 8)	Group I (n = 35)	Group II (n = 30)
Acetic acid (C2)	0.013 ± 0.006	0.090 ± 0.030*	0.089 ± 0.040
Propionic acid (C3)	0.146 ± 0.002	0.063 ± 0.020*'	0.040 ± 0.010*'
Butyric acid (C4)	0.065 ± 0.02 3	0.130 ± 0.019*	0.090 ± 0.033
∑(C2-C4)	0.093 ± 0.028	0.207 ± 0.045*	0.185 ± 0.060

*: P < 0.05; **: P < 0.01 – significance of the differences according to t-criterion in comparison with the control group.

Table 3. Deviations in the colon microflora composition in NAFLD children depending on the GB function

Microorganisms	Amount of microorganisms (Me (Q1; Q2)), lg CFU/g		
	Control group (n = 8)	Group I (n = 35)	Group II (n = 30)
Bifidobacterium spp.	9.5 (9.5; 9.5)	9.0 (9.0; 9.5)	9.3 (9.0; 9.5)
Lactobacillus spp.	6.9 (6.5; 8.4)	2.6 (0; 6.0)*	5.05 (0.0; 6.5)**
Candida spp.	1.04 (0.0; 1.8)	2.9 (0; 4.7)	2.3 (0.0; 6.0)
Enterobacterales:			
Enterococcus spp.	8.1 (6.5; 9.0)	6.1 (6.1; 8.1)*	8.4 (7.7; 9.0)**
Citrobacter spp.	–	2.2 (0.0; 5.0)	0.5 (0.0; 4.9)**
Proteus spp.	–	–	0.8 (0.0; 1.8)
Klebsiella spp.	–	–	2.2 (0.0; 3.6)
Escherichia coli with normal enzymatic activity	8.5 (8.2; 9.0)	8.4 (7.7; 8.7)	9.0 (8.4; 9.0)
Escherichia coli hemolytic	–	1.5 (0.0; 8.4)	–
Escherichia coli lactose negative	0.67 (0.0; 1.6)	1.7 (0.0; 3.1)	2.5 (0.0; 6.6)*
Staphylococcus aureus	–	2.7 (0.0; 5.7)	2.0 (0.0; 4.9)
Staphylococcus epidermidis	0.8 (0.0; 2.0)	1.4 (0.0; 2.4)*	1.3 (0.0; 6.9)
Staphylococcus saprophyticus	0.5 (0.0; 1.9)	1.2 (0.0; 2.9)	1.1 (0.0; 1.9)

*: P < 0.05 – significance of the differences according to the U-criterion in comparison with the control group; **: P < 0.05 – significance of the differences according to the U-criterion in comparison with group I.

GB normokinesia (40.0 %) without significant differences between groups (*Fig. 1*). Lactose malabsorption was also observed more often in NAFLD children with impaired contractile GB function accounting for 51.4 % of cases.

Among children with identified SIBO, a single-humped curve was observed that was characterized by an early (at the 60th minute) increase in the hydrogen concentration up to 22.18 ± 2.99 ppm in 9 (56.2 %) NAFLD patients with hypokinesia.

There was a significant increase in the hydrogen concentration at the 90th minute up to 59.71 ± 3.36 ppm and 47.57 ± 3.97 ppm (P < 0.05), in patient groups I and II, respectively, with the further gradual decline at the 120th, 150th and 180th minutes (*Table 1*). There was evidence of lactose malabsorption with SIBO in the small intestine with impaired function of the ileocecal valve in obese patients regardless of GB function (P < 0.05).

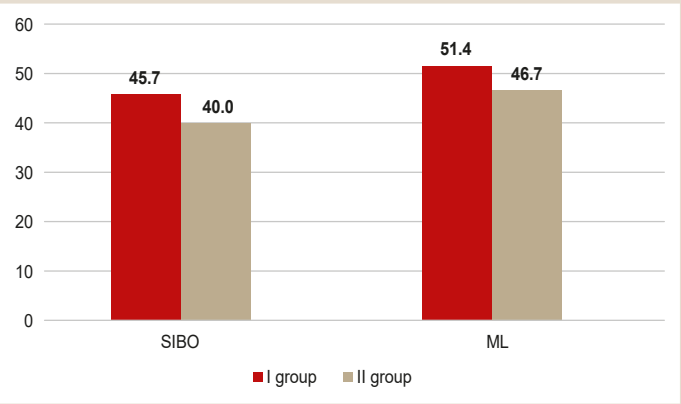

Fig. 1. The frequency of SIBO and lactose malabsorption detection in children with NAFLD depending on the gallbladder function.

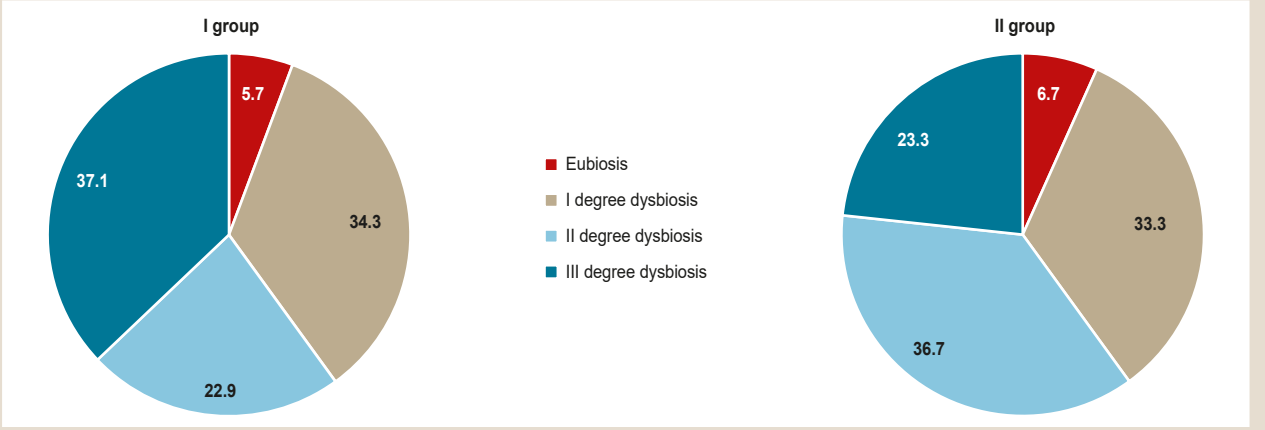

Fig. 2. Dysbiosis grade distribution in NAFLD children depending on the GB function.

Analysis of the fecal SCFA spectrum in NAFLD children showed 2.0 times ($P < 0.05$) increase in the butyric acid concentration in children with GB hypokinesia compared to the control group. There was also 6.9 times ($P < 0.05$) increase in the acetic acid concentration in group I children and 6.8 times ($P < 0.01$) in group II children as compared to the control group (*Table 2*).

At the same time, a significant decrease in the fecal propionic acid content was revealed in children with GB hypokinesia by 2.4 times ($P < 0.001$) and in children with GB normokinesia by 3.6 times ($P < 0.001$) compared to the control group. Due to the increased content of fecal acetic and butyric acids, the total content of SCFA (Σ (C2-C4)) was 2.3 times increased in group I children compared to that in the control group ($P < 0.05$).

Microbiological assessment showed a decrease in the *Bifidobacterium spp*. level in 6 (17.1 %) group I patients and in 5 (16.7 %) group II patients. Also, a decrease in the concentration of *Lactobacillus spp*. was found in 31 (88.6 %) group I children and in 21 (70.0 %) group II children.

Opportunistic *Enterobacterales* of the *Citrobacter spp*. were identified during the examination in 8.6 % of group I patients and in 3.3 % of group II patients. Moreover, opportunistic *Enterobacterales* of the *Proteus spp*. (3.3 % of patients) and *Klebsiella spp*. (26.7 % of patients) were found only in group II children. In almost half of the patients, this concentration was ℓg 7.5 – ℓg 8.9 CFU/g. An increased concentration of pathogenic *Staphylococcus aureus* was found in 20.0 % of group I patients and in 16.7 % of group II patients. An increased concentration of yeast-like fungi of the genus *Candida* was prevailed in 37.1 % of group I patients and in 16.7 % of group II patients. In addition, hemolytic *Escherichia coli* biovars were sown in 17.1 % of group I children. Their dominance over *Escherichia coli* with normal enzymatic activity was observed in most cases.

The quantitative changes in the identified colonic microbiota deviations in NAFLD children are presented in *Table 3*.

As can be seen from *Table 3*, the concentration of *Lactobacillus spp*. in children with GB hypokinesia was 1.9 times ($P < 0.05$) significantly lower than that in children with GB normokinesia. Furthermore, the amount of *Enterococcus spp*. in group I children was significantly 1.3 times ($P < 0.05$) lower than that in the control group and 1.4 times ($P < 0.05$) – in group II children.

Differences in dysbiosis grade distribution were found in group I children, decompensated form of dysbiosis was most often diagnosed (37.1 % of children) while subcompensated form of dysbiosis was dominated in group II (36.7 % of patients) (*Fig. 2*). Deviations in the microbiota composition were caused by a sharp decrease in the number of the main species of colonic microbiota and an increase in the concentration of opportunistic microflora.

Correlation analysis demonstrated that the presence of SIBO was associated with dysbiosis degree ($r = 0.512$, $P < 0.01$). In children with GB hypokinesia, the concentration of *Candida spp*. was positively correlated with the content of lactose-negative *Escherichia coli* ($r = 0.446$; $P = 0.029$) and opportunistic *Enterobacterales* ($r = 0.773$; $P = 0.003$), *Staphylococcus aureus* ($r = 0.597$; $P = 0.002$). Moreover, a relationship was found between the sphincter of Oddi dysfunction presence and the content of *Lactobacillus spp*. ($r = -0.510$; $P = 0.025$); the frequency of sludge detection and the content of *Enterococcus spp*. ($r = 0.453$; $P = 0.026$); the frequency of inhomogeneous GB content detection and the level of *Staphylococcus aureus* ($r = 0.69$; $P < 0.0001$) and the content of *Candida spp*. ($r = 0.41$; $P = 0.045$); the frequency of *Proteus spp*. detection and the level of acetic acid ($r = -0.480$; $P < 0.05$).

Discussion

In recent years, the role of intestinal microbiota in the development of NAFLD has been actively studied. It was shown that intestinal microbiota plays an important role in the development of metabolic changes in NAFLD [4,9,10]. Some studies indicate that the development of NAFLD is associated with significant changes in microbiota composition and an increase in the level of bacterial endotoxins in both adults [14] and children [4]. Safari's Z. studies found a higher abundance of *Enterobacteriaceae* and the genus *Escherichia*, *Ruminococcus* in NAFLD patients at late stages of fibrosis [15]. Our study also has demonstrated the presence of changes in the qualitative and quantitative composition of the colon microbiota in 94.3 % of NAFLD children with GB hypofunction and in 93.3 % of NAFLD

children with normal GB contractile function. Changes in the microflora of the small intestine as SIBO were observed in more than half of NAFLD children.

In NAFLD children with GB hypokinesia, according to our study data, the level of fecal acetic acid was increased by 6.9 times ($P < 0.05$), and butyric acid – by 2.0 times ($P < 0.05$) compared to the control group. Such changes in SCFA levels in obese NAFLD children was associated with higher grades of intestinal dysbiosis. These metabolites act as mediators between the intestinal microbiota and the whole body to regulate intestinal permeability, control inflammation and metabolism of bile acids as well as immune functions. Our study also has found the decrease in fecal propionic acid in NAFLD children regardless of GB function that may predispose to microcirculation impairment in the intestinal mucosa and metabolic dysregulation since propionic fatty acid is an indirect regulator of the lipid metabolism and reduces the effect of blocking opportunistic microflora attachment to colonocytes [16].

Thus, a decrease in the GB contractile function in NAFLD children is associated with the sharp decrease in the number of the main intestinal microbiota symbionts, increase in the concentration of opportunistic microflora and production of acetic and butyric SCFA. These qualitative and quantitative changes in the composition of intestinal microbiota, an imbalance in the spectrum of fecal SCFA are powerful mutually potential factors that may worsen the course of NAFLD in children.

Conclusions

1. Lactose-dependent SIBO was revealed in almost half (42.9 %) of NAFLD children regardless of the GB function.

2. The concentrations of *Lactobacillus spp*. and members of the *Enterobacterales* family in colonic content of NAFLD children with GB hypofunction were significantly lower (by 1.9 times ($P < 0.05$) and 1.4 times ($P < 0.05$), respectively), than those in NAFLD children with normal GB contractile function as well as decompensated dysbiosis were observed more often (37.1 % of cases).

3. In NAFLD children with GB hypokinesia, the significant increase in the concentration of fecal acetic acid by 6.9 times ($P < 0.05$), butyric acids by 2.0 times ($P < 0.05$) was observed, which may suggest the involvement of bile acids in the microbiome composition regulation.

Perspectives for further research. The study on the relationship between biliary dysfunction and intestinal microbiocenosis disorders, as well as the influence of the intestinal microflora on the metabolic disturbances progression and structural and functional changes in the hepatobiliary tract in children, would help to develop a multi-target strategy for the management of this patient group. It will improve the quality of life and the efficiency of the treatment for patients, reduce cardiovascular risk and improve the prognosis of the NAFLD course, reduce the costs associated with the treatment and rehabilitation of these patients.

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