The aim of the study – to explore the gut microbiota composition and its association and correlational relationship with biochemical factors in patients with nonalcoholic fatty liver disease (NAFLD) compared to the patients without liver disease.

Materials and methods. 154 patients with NAFLD and 83 controls were included into the study. The examination involved the biochemical parameters, ultrasound data, elastography, identification of gut microbial composition by real-time PCR. Patients of both groups were matched by patients characteristics.

Results. There was higher level of Bacteroidetes in group with NAFLD. Actinobacteria range and Firmicutes/Bacteroidetes index (F/B index) in patients with NAFLD was above in comparison with patients of control group. Age and BMI are the risk factors for NAFLD development. Actinobacteria in NAFLD group positively correlated with Tumor necrosis factor alpha (α), while F/B index was in strong relationship with ALT, TG, VLDL. The F/B index was in negative correlational relationship with TNF-α in group without liver injury.

Conclusions. The composition of the intestinal microbiota is different between group of patients with NAFLD and controls. Actinobacteria and F/B index growth could be discussed as one of the risk factor for the fatty infiltration development in patients with NAFLD, taking to account the fact that F/B index could be associated with such inflammation markers as ALT and TG. Simultaneously, the same microbiome phyла could be the potential prevention factor of lipid disorders in patients without liver injury.
Introduction
Nonalcoholic fatty liver disease (NAFLD) is a liver disease that affects about 25-30 % of the population in the developed countries. The development of NAFLD is significantly influenced by genetic and environmental factors [1]. Otherwise, more and more evidence points to a close relationship between the composition of microbiota and the development and progression of NAFLD and its complications. The liver is first exposed to contact with products such as bacterial endotoxins, mainly lipopolysaccharide (LPS), unmethylated DNA sequences that, when released into the liver, may trigger an inflammatory reaction that affects the course of some hepatic disorders [2]. Deriving from its anatomical position, the liver receives 70 % of its blood supply from the intestine through the portal vein, so it represents the first line of defense against gut-derived antigens, and one of the most exposed organs to gut-derived toxic factors, such as bacteria and bacterial by-products [3]. Thus, the portal vein supplies the liver with blood rich in digestive products, but also microbiological components derived from the microbiota. Additionally, endotoxemia can affect insulin resistance in NAFLD and exert its hepatotoxic action through a compromised intestinal barrier [4].

The gut microbiota is now considered as a major metabolic internal organ, composed of >10^{14} microorganisms and containing a second genome (named the metagenome), which is up to 100-400 times that of humans. It consists of mainly anaerobic bacteria, of which 60-90 % belong to two types: Bacteroidetes and Firmicutes [5]. The intestinal microbiota is influenced by diet, gastrointestinal infections, drugs used, mainly antibiotics, age, body weight, past surgery and coexisting diseases [6]. The intestinal microbiota is involved in the metabolism of the host by the secretion of bioactive metabolites, which affects the immune system and permeability of the mucosal barrier. It takes part in the fermentation of undigested food residues, the synthesis of vitamin K and B group vitamins and in the synthesis of short-chain fatty acids (SCFAs), which are the source of energy for intestinal cells. The gut microbiota may affect all risk factors for the development of NAFLD by disturbing energy homeostasis, enhancing IR, increasing oxidative stress, developing inflammation, and evoking alteration of bile acids and choline levels [7]. Most studies have shown that the levels of Firmicutes are increased whereas those of Bacteroidetes are decreased in obesity and its related diseases in humans as well as rodents. Current evidence suggests that the increased Firmicutes/Bacteroidetes ratio is a potential phenotype of obesity [8].

Finding novel mechanisms for the pathogenesis of NAFLD, in particular involving the microbiota, could emphasize new research areas to develop new therapeutic targets.

The aim of study
To explore the gut microbiota composition and its association and correlational relationship with biochemical factors in patients with NAFLD compared to the patients without liver disease.

Materials and methods
The study involved 154 patients of main group with nonalcoholic fatty liver disease (72 men and 82 women) aged from 23 to 77 years (average age 46.64±2.52 years) which were engaged to the ambulatory screening in «Medicover Ukraine» (Lviv, Ukraine) and «Agency Truskavetskuryort» (Truskavets, Ukraine) during 2018-2020 years. The diagnosis of fatty liver infiltration was based on liver transient elastography and ultrasound examination. Likewise, the control group involved 83 almost healthy control subjects (38 men and 45 women) aged from 25 to 55 years (an average 32±1.54 years) with normal liver size, structure and parenchyma echogenicity by transient elastography.

The average waist circumference in main group was 94.6±1.09 cm (in men), 88.3±0.65 cm (in women), in controls – 91.2±2.1 cm in men, 83.6±1.8 in women. The exclusion criteria for patients in this study were history of significant alcohol consumption (> 20 g/day), evidence of hepatitis B or C infection, autoimmune hepatitis, histological evidence of other concomitant chronic liver diseases, pregnant women, cirrhosis with and without complications (ascites, variceal bleeding, systemic infection, or hepatocellular carcinoma), history of chronic inflammatory bowel disease or bariatric surgery, or treatment with antibiotics within 1 month before inclusion.

Biochemical tests. Both groups of patients underwent biochemical evaluation of serum that included blood cell count, lipid profile (total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), triglycerides (TG), C-reactive protein (CRP), alanaminotransferase (ALT), aspartaminotransferase (AST), gamma-glutamyl transpeptidase (GGTP), bilirubin (total, direct, indirect), urea, uric acid, albumin, total protein, tumor necrotizing factor-α (TNF-α), apolipoprotein B (apo B), apolipoprotein A1 (apo A1), HOMA index. Biochemical tests were carried out using commercially available test kits.

The ultrasound method as additional for the liver elastography included the next signs for steatosis as diffuse increase in the echogenicity of the liver parenchyma, decreased attenuation on the liver and the ratio between the brightness level of the liver and the right kidney, that was calculated for the hepato-renal index (HRI) determination [9].

Sample collection and DNA extraction. Fresh stool samples were provided by each subject in a stool sample container on site. Within 10 min upon defecation, the fecal sample was aliquoted and aliquots were immediately stored at 20 °C for 1 week until DNA isolation. DNA was extracted from 1.5-2 frozen stool aliquots using the phenol-chloroform method by protocol. DNA was finally eluted in 200 μl elution buffer. The DNA quantity and quality was measured by NanoDrop ND-8000 (Thermo Scientific, USA). Samples with a DNA concentration less than 20 ng or an A 260/280 less than 1.8 were subjected to ethanol precipitation to concentrate or further purified, respectively, to meet the quality standards.

Oligonucleotide primers. Quantification of different taxa by qPCR using primers targeting the 16S rRNA gene, specific for Firmicutes, Actinobacteria and
Identification of microbial composition. Determination of microbial composition at the level of major microbial phyla was carried out by identification of total bacterial DNA, and DNA of Bacteroidetes, Firmicutes and Actinobacteria was performed with quantitative real-time PCR (qRT-PCR), using gene-targeted primers.

Statistical analysis. The reliability of changes in indicators in the normal distribution in the sample was determined by the paired Student’s t test, in case of difference from the normal – by the criterion of F. Wilcoxon. Differences were considered statistically significant for p<0.05. The data distribution was investigated by the graphical method and by the Shapiro-Wilk normality test. Correlational relationship was examined by calculating Pearson’s product moment correlation coefficients (r) on raw data. All calculations charting were carried out in the programming language R in the development environment of RStudio.

Results and Discussion
Biochemical and anthropometric charasteristics of patients included into the study is given below in Table 1.

<table>
<thead>
<tr>
<th>Variables (mean±SD)</th>
<th>Main (n=154)</th>
<th>Control (n=83)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>46.64±2.52</td>
<td>32±1.54</td>
<td>*0.0000006088</td>
</tr>
<tr>
<td>BMI</td>
<td>27.61±0.73</td>
<td>23.37±0.7</td>
<td>*0.002169</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>6.2±0.21</td>
<td>5.9±0.28</td>
<td>0.2089</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>2.2±0.36</td>
<td>1.12±0.09</td>
<td>*0.000689</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.3±0.06</td>
<td>1.52±0.07</td>
<td>*0.01144</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.98±0.17</td>
<td>3.58±0.23</td>
<td>*0.00979</td>
</tr>
<tr>
<td>VLDL, mmol/L</td>
<td>1.09±0.16</td>
<td>0.77±0.07</td>
<td>0.2089</td>
</tr>
<tr>
<td>HOMA</td>
<td>3±0.2</td>
<td>1.55±0.04</td>
<td>0.07143</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.3±0.28</td>
<td>5.05±0.10</td>
<td>0.108</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.28±0.06</td>
<td>1.01±0.06</td>
<td>0.1649</td>
</tr>
<tr>
<td>Apo A</td>
<td>1.47±0.04</td>
<td>1.39±0.03</td>
<td>0.5678</td>
</tr>
<tr>
<td>ALT</td>
<td>35.99±5.32</td>
<td>32.65±6.09</td>
<td>0.2558</td>
</tr>
<tr>
<td>AST</td>
<td>27.02±3.01</td>
<td>23.9±2.23</td>
<td>0.2615</td>
</tr>
<tr>
<td>GGTP</td>
<td>40.82±5.65</td>
<td>31.45±3.58</td>
<td>0.4519</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>11.7±1.31</td>
<td>17.16±1.78</td>
<td>*0.01851</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>3.31±0.30</td>
<td>3.64±0.40</td>
<td>0.7049</td>
</tr>
<tr>
<td>Indirect bilirubin</td>
<td>9.00±1.09</td>
<td>13.41±1.74</td>
<td>0.1009</td>
</tr>
<tr>
<td>CRP</td>
<td>4.01±0.80</td>
<td>2.28±0.29</td>
<td>0.3692</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5.90±0.39</td>
<td>4.43±0.02</td>
<td>0.1642</td>
</tr>
<tr>
<td>Uric acid</td>
<td>369.57±16.46</td>
<td>322.40±15.04</td>
<td>0.1738</td>
</tr>
<tr>
<td>Urea</td>
<td>5.47±0.37</td>
<td>4.04±0.14</td>
<td>*0.008276</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>74.59±3.75</td>
<td>69.40±4.28</td>
<td>0.469</td>
</tr>
<tr>
<td>Albumin</td>
<td>45.24±0.88</td>
<td>46.33±1.20</td>
<td>0.9818</td>
</tr>
<tr>
<td>Total protein</td>
<td>71.56±0.84</td>
<td>72.52±1.08</td>
<td>0.8189</td>
</tr>
</tbody>
</table>

Table 1

Due to the results, the range of LDL was significantly higher in patients with NAFLD than in controls. HDL in control group exceeded the level in main. TG were higher in patients with NAFLD with significance of difference 0.000689. BMI was higher in the main group (p=0.0002169).

The microbiota composition was evaluated in both groups, where the main phylum of bacteria were explored – Bacteroidetes, Firmicutes, Actinobacteria, Firmicutes/Bacteroidetes ratio. The results of analysis are presented in the Fig. 1.

According to this picture, the level of Bacteroidetes in controls was significantly higher than in patients with NAFLD. Actinobacteria range in patients with NAFLD was above than in patients of control group. F/B index exceeded in patients with NAFLD. The significant difference in the age of patients was not the reason to mark the groups of patients as nonrelevant. As we see in different data – age is one of the risk factor for the NAFLD development. Thus, the age of patients without fatty infiltration could be less and is not the reason to change the design of study.

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Moreover, one of the important points was paid
to the correlational relationship between the age and
NAFLD presence in the merged group of all patients. It
was r=0.54 and showed that NAFLD is associated with
age in our study too. Thus, this point has to be marked
as the risk factors of fatty infiltration. Consequently,
the groups are relevant to each other and could be in
comparison.

Taking into account the results, it was interesting to
pay attention to the correlational relationship between
different phylum of bacteria and biochemical markers not
only in patients with NAFLD, but also in controls (Fig. 2).

![Fig. 1. The composition of intestinal microbiota in patients with NAFLD and controls.](image)

The negative correlational relationship was marked
in NAFLD group between Bacteroidetes and Firmicutes
(r= -0.68), Bacteroidetes and Actinobacteria (r= -0.56),
Bacteroidetes and F/B index (r= -0.056).

Actinobacteria in NAFLD group positively correlated
with TNF-α (r=0.41, p<0.05), F/B index was in strong
relationship with ALT (r=0.61, p<0.05), TG (r=0.53,
p<0.05), VLDL (r=0.4, p<0.05).

In controls Bacteroidetes strongly correlated with
Firmicutes (r= -0.93), Actinobacteria (r= -0.84), F/B
index (r= -0.88). Actinobacteria was in correlation with
Apo-A1 (r=0.74), Apo-B (r= -0.74). The correlational
relationship was marked between Bacteroidetes and
Apo-A1 (r= -0.43), Apo-B (r=0.44). F/B index was in
strong correlation with Apo-B (r= -0.61), apo-A1 (r=0.6)
and in negative correlation with TNF-α.

In 1921, B. Hoefert first drew attention to the
significant changes in the composition of the intestinal
microbiota in patients with chronic liver disease [10].

The traditional theory of two hits in the pathogenesis
of NAFLD is currently being developed and replaced
with the multiple parallel hits hypothesis, describing
NAFLD as a result of the action of many factors acting

![Fig. 2. The correlational relationship between microbiome and biochemical markers in patients with NAFLD.](image)
First of all, hyperinsulinemia, which is a well-known trigger of hepatic fibrosis progression. Insulin resistance leads to an increase in levels of free fatty acids (FFAs) by up-regulation of their hepatic synthesis and enhancement of adipose tissue lipolysis. When the level of FFAs exceeds the possibility of their transformation and transport to peripheral tissues (as very low density lipoprotein – VLDL), oxidative stress occurs, which leads to alteration in the adipokine profile, increased production of pro-inflammatory cytokines (TNF-α, IL-6) and, consequently, to the development of fatty infiltration [12, 13].

The human body functions in close relationship with bacteria that predominantly inhabit the gastrointestinal tract to produce essential amino acids, vitamins and digesting plant polysaccharides. The majority of healthy gut microbiota comprises Bacteroidetes and Firmicutes; their ratio has been found altered in human and mice obesity. Many studies have explored the possible causes of metabolic syndrome occurrence in its co-morbidities, including NAFLD [14, 15].

Various studies show not entirely consistent data on the bacteria that predominate in gut microbiota. Among patients with obesity, a decrease in the number of bacteria was found from the phylum Bacteroidetes, and the rise of Firmicutes. Similarly, a reduced percentage of Bacteroidetes was observed in patients with NASH when compared to those with simple steatosis or without liver injuring [16]. Another paper by Boursier et al., in which 57 patients with biopsy proven NAFLD were examined, confirmed a definite increase in the number of Bacteroidetes in patients with NASH and the growth of Ruminococcus bacteria (phylum Firmicutes) in patients with existing liver fibrosis and reduction of Prevotella bacteria (Bacteroidetes type) in patients with NASH [17]. However, there were also studies in which no significant differences in the number of types of bacteria were found [18].

Gut microbiota may stimulate hepatic fat deposition and promote NASH though several mechanisms:
1. It promotes obesity by improving energy yield from food
2. It regulates gut permeability, low-grade inflammation and immune balance
3. It modulates dietary choline metabolism
4. It regulates bile acid metabolism
5. It increases endogenous ethanol production by bacteria

Systemic increase of hepatic expression of TNF-α correlates with circulating high levels of LPS binding protein in patients with NAFLD, and leads to a greater extent in patients with NASH [19, 20].

In our study the Actinobacteria growth was the threatening factor for those patients who were diagnosed with NAFLD by its further possible correlation with TNF-α increasing, that is the potential marker of steatohepatitis. Also, F/B that exceeded in patients with NAFLD was in positive correlation with such markers as ALT (r=0.61), TG (r=0.53), VLDL (r=0.4). Taking into account such factors we can suggest that separate bacteria phyla in gut microbiota could be the potential markers of fatty infiltration development.

The difference in the composition of the microbiota in patients with NAFLD and patients in the control group is important, as it gives reason to believe that the level of bacteria in NAFLD, especially those bacteria that are aggressive and provoke pro-inflammatory and dysmetabolic processes, will get the chance to be corrected by targeted probiotic therapy or fecal transplantation methods in the nearest future [21].

It is interesting, that the same gut bacteria – F/B and Actinobacteria – have been reduced the proatherogenic Apo-B and increased the protective Apo-A1 factors in patients without NAFLD. Thus, we can hypothesize that the microbiome could provoke the development of fatty
infiltration in case of NAFLD presence. Vice versa, the same bacteria could be the protective factor in patients without liver injury and prevent the increasing of factors that are leading to the lipid metabolism violation. The same bacteria manifest themselves in different ways—aggressively or protectively in the presence or absence of fatty steatosis. Probiotic therapy should be selected taking into account this factor and may differ depending on whether fatty infiltration is present.

Conclusions
1. **Actinobacteria** range and F/B index in patients with NAFLD was above than in patients of control group. **Bacteroidetes** level in controls was significantly higher than in patients with NAFLD.
2. The **Actinobacteria** growth in patients with NAFLD could provoke TNF-α increasing—one of the main factors of nonalcoholic steatohepatitis development.
3. F/B index is associated with such markers of liver injury as ALT and TG.
4. **Actinobacteria** and F/B index growth could be discussed as one of the markers for fatty infiltration development in patients with NAFLD.

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