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Structure and Metabolic Activity of the Gut Microbiota in Diarrhea-Predominant Irritable Bowel Syndrome Combined with Functional Dyspepsia

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Abstract: Gut dysbiosis presents in many digestive diseases. The aim of this study is to investigate the composition of the gut microbiota and its metabolic activity in patients with diarrhea-predominant irritable bowel syndrome combined with functional dyspepsia (I + D). This study included 60 patients with I + D and 20 healthy controls. Gut microbiota composition was studied using 16S rRNA gene sequencing. The short-chain fatty acids (SCFAs) spectrum was determined via gas–liquid chromatography. Patients with I + D had an increase in the abundance of *Holdemanella*, Erysipelotrichaceae, Erysipelotrichales, Prevotellaceae, *Agathobacter*, *Slackia*, *Lactococcus*, Pseudomonadaceae, *Stenotrophomonas*, Xanthomonadaceae, Rhizobiaceae, Erysipelatoclostridiaceae, Lachnospiraceae, and other taxa in addition to a decrease in the abundance of *Frisingicoccus*, *Ralstonia*, Burkholderiaceae, *Hungatella*, *Eisenbergiella*, *Parabacteroides*, Peptostreptococcaceae, *Merdibacter*, *Bilophila*, Rikenellaceae, Tannerellaceae, Bacteroidaceae, and *Flavonifractor* in comparison to controls. Patients with I + D showed significantly higher total SCFA content in feces; increased absolute content of acetic acid, propionic acid, butyric acid, and isoacids; and a significant negative shift in the anaerobic index. The relative levels of the main SCFAs and isoacids in the patient group did not differ significantly from those in the control group. The fecal acetate and isoacid levels correlated with the severity of diarrhea. The fecal butyrate level correlated with the severity of flatulence.

Keywords: irritable bowel syndrome; functional dyspepsia; short-chain fatty acids; gut microbiota; gut microbiome

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1. Introduction

Irritable bowel syndrome (IBS) is a functional bowel disease in which recurrent abdominal pain is associated with defecation or a change in bowel habits [1]. Depending on the prevailing disorders, the following variants of IBS are distinguished: with predominant diarrhea (IBS-D), with predominant constipation, mixed, and unclassified [1]. Functional dyspepsia (FD) is a medical condition that significantly impacts on the usual activities of a patient and is characterized by one or more of the following symptoms: postprandial fullness, early satiation, epigastric pain, and epigastric burning that are unexplained after a routine clinical evaluation [2]. In both cases, the absence of any organic digestive disease that could explain the presence of these symptoms is implied. The exact criteria for these diseases are defined in the latest Rome IV criteria [1,2]. The worldwide prevalence of IBS is 11.2% [1], and the prevalence of FD varies from 10% to 30% of the population [2]. Many individuals have overlapping IBS and FD (13–87%) [3].

The gut microbiota is a complex of microorganisms that colonizes the small and large intestines. The human gut microbiota is dominated by the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. According to one study, Firmicutes normally account for 80% of bacteria in the gut microbiota, followed by Bacteroidetes (approximately 10%), Actinobacteria (1.5%), and Proteobacteria (approximately 1%); less than 10% of the bacteria belong to other phyla [4]. The gut microbiota in the human body performs several functions.

The protective function of the microbiota is to maintain the immune response and create colonization resistance, which prevents the action of pathogenic and opportunistic pathogenic bacteria on the mucoepithelial barrier of the gastrointestinal tract. An increase in the permeability of the mucoepithelial barrier leads to increased adhesion of potential and overt pathogenic bacteria to the intestinal wall, which in turn causes nonspecific inflammation [5–7]. Minimal inflammation is observed in patients with irritable bowel syndrome (IBS) and functional dyspepsia (FD) [8].

Metabolic function refers to the ability of bacteria to ferment dietary fibers (polysaccharides) with the formation of short-chain fatty acids (SCFAs), utilize hydrogen, and synthesize amino acids, vitamins, and antimicrobial substances [9]. The main SCFAs include acetic, propionic, and butyric acids. A change in the composition of the gut microbiota inevitably affects its metabolic activity, which may promote gastrointestinal tract dysfunction and aggravate the clinical symptoms of IBS.

Numerous studies have described changes in the intestinal microbiota composition in patients with IBS compared to that in healthy individuals [10]. Results demonstrate that in patients with IBS, including those with IBS-D, there are changes in the level and spectrum of SCFAs [10–12]. However, the SCFA profile and gut microbiota state in patients with other functional gastrointestinal diseases, including FD, have not been studied. In addition, despite the availability of data on the SCFA profile and gut microbiota composition in IBS (in particular, IBS-D), these indices have not been analyzed simultaneously in a single study. Accordingly, the aim of the current study was to evaluate and compare the gut microbiota composition and SCFA profiles in patients with IBS-D combined with FD (I + D) with those in healthy control subjects.

2. Results

There were no significant differences between the tested patients and healthy controls in terms of age and sex distribution. The mean age was 33.75 $\pm$ 9.52 years in the patient group and 39.13 $\pm$ 15.20 in the control group (p = 0.195). The sex ratio (m/w) in the patient group was 23/37 in the control group and 5/15 in the experimental group (p = 1.000). The housing, other living conditions and diet of the controls were similar to those of the patients.

All patients and controls had normal complete blood counts and biochemical blood analysis data. Gastrointestinal Symptom Rating Scale (GSRS) parameters represented in Table 1.

Table 1. Symptoms of digestive disorders according to Gastrointestinal Symptom Rating Scale (GSRS) in patients with diarrhea-predominant irritable bowel syndrome and functional dyspepsia combination (I + D) and in healthy individuals from the control group.

Symptom	I + D (n = 60)	Control (n = 20)	p
Pain or discomfort in upper abdomen	2 [1–3]	1 [1–1]	<0.001
Heartburn	2 [1–3]	1 [1–1]	<0.001
Acid reflux	1 [1–3]	1 [1–1]	0.002
Hunger pangs	1 [1–3]	1 [1–1]	0.002
Nausea	2 [1–3]	1 [1–1]	<0.001

Table 1. *Cont.*

Symptom	I + D (*n* = 60)	Control (*n* = 20)	*p*
Rumbling	4 [3–5]	1 [1–1]	<0.001
Stomach felt bloating	3 [3–5]	1 [1–1]	<0.001
Burping	2 [2–4]	1 [1–1]	<0.001
Passing gas or flatus	3 [2–4]	1 [1–1]	<0.001
Constipation	1 [1–1]	1 [1–1]	0.116
Increased stool frequency	3 [2–4]	1 [1–1]	<0.001
Loose stool	4 [3–5]	1 [1–1]	<0.001
Hard stool	1 [1–2]	1 [1–1]	0.056
Urgent need to have a bowel movement	1 [1–2]	1 [1–1]	<0.001
Sensation of incomplete bowel emptying	2 [2–3]	1 [1–1]	<0.001

2.1. Gut Microbiota Data

Patients with IBS-D and FD did not show any significant difference in the abundance of bacteria phyla and classes and in the alpha-diversity indices of the gut microbiota in comparison to healthy controls (Table 2).

Table 2. Comparative assessment of the abundance (reads) of the main bacterial phyla and classes and alpha-diversity indices of fecal microbiota in patients with diarrhea-predominant irritable bowel syndrome and functional dyspepsia (I + D) and in healthy individuals from the control group.

Taxon/Index	Taxon Rank	I + D (*n* = 60)	Control (*n* = 20)	*p*
Shannon index	-	4.52 [4.08–4.83]	4.54 [4.43–4.79]	0.562
Chao1 index	-	567 [456–758]	600 [477–690]	1.000
ACE index	-	554 [455–746]	589 [469–680]	0.918
Firmicutes	phylum	83,560.2	70,600.2	0.075
Bacteroidota	phylum	13,721.4	27,140.8	0.075
Actinobacteriota	phylum	3733.0	4357.4	0.411
Proteobacteria	phylum	2410.6	2739.5	0.570
Verrucomicrobiota	phylum	628.7	138.8	0.340
Desulfobacterota	phylum	98.8	137.6	0.189
Clostridia	class	66,610.1	64,506.2	0.632
Bacilli	class	14,653.1	3373.5	0.102
Bacteroidia	class	13,719.2	27,140.7	0.115
Actinobacteria	class	3147.1	3317.0	0.434
Gammaproteobacteria	class	2339.4	2712.1	0.678
Negativicutes	class	2157.1	2683.9	0.737
Verrucomicrobiae	class	628.7	138.6	0.434
Coriobacteriia	class	583.6	1040.1	0.853

At the levels of orders, families, and genera, patients with I + D showed an increase in the abundance of *Holdemanella*, Erysipelotrichaceae, Erysipelotrichales, Prevotellaceae, *Agathobacter*, *Slackia*, *Lactococcus*, Pseudomonadacea, *Stenotrophomonas*, Xanthomonadaceae, Rhizobiaceae, Erysipelatoclostridiaceae, Lachnospiraceae, and other taxa in addition to a decrease in the abundance of *Frisingicoccus*, *Ralstonia*, Burkholderiaceae, *Hungatella*, *Eisenbergiella*, *Parabacteroides*, Peptostreptococcaceae, *Merdibacter*, *Bilophila*, Rikenellaceae, Tannerellaceae, Bacteroidaceae, and *Flavonifractor* in comparison to healthy individuals (Table 3).

There was no significant correlation between the abundance of gut microbiota taxa on the one hand and drugs used, GSRS and laboratory parameters on the other.

Table 3. Comparative assessment of the abundance (reads) of gut bacterial orders, families, and genera in fecal samples in patients with diarrhea-predominant irritable bowel syndrome and functional dyspepsia (I + D) and in healthy individuals from the control group (only significant differences are shown).

Taxon	Taxon Rank	LogFC	I + D (n = 60)	Control (n = 20)	p
Holdemanella	genus	9.01	6183.8	0.0	<0.001
Erysipelotrichaceae	family	5.02	7294.1	212.9	0.035
Prevotellaceae	family	2.88	1956.4	254.5	0.036
Erysipelotrichales	order	2.70	9965.0	1527.8	0.001
Agathobacter	genus	2.28	7555.1	1547.1	0.003
Slackia	genus	2.23	44.4	0.0	0.009
Lactococcus	genus	1.84	40.9	2.7	0.011
Pseudomonadales	order	1.73	144.8	35.3	0.006
Pseudomonadaceae	family	1.69	139.5	35.1	0.013
Pseudomonas	genus	1.67	137.1	34.9	0.009
Stenotrophomonas	genus	1.60	76.4	17.1	0.003
Xanthomonadaceae	family	1.60	77.1	17.4	0.003
Xanthomonadales	order	1.60	77.1	17.4	0.001
Rhizobiaceae	family	1.10	28.4	6.8	0.036
Erysipelatoclostridiaceae	family	1.02	2669.3	1314.8	0.039
Lachnospiraceae	family	0.33	32,140.1	25,607.6	0.035
Lachnospirales	order	0.33	32,154.7	25,619.3	0.02
Flavonifractor	genus	−1.07	68.5	156.4	0.039
Bacteroidaceae	family	−1.15	9830.4	21,868.1	0.036
Tannerellaceae	family	−1.33	590.3	1501.8	0.036
Rikenellaceae	family	−1.42	893.9	2404.9	0.047
Bilophila	genus	−1.46	42.3	137.4	0.009
Merdibacter	genus	−1.49	6.2	39.3	0.003
Peptostreptococcaceae	family	−1.51	332.4	971.8	0.044
Parabacteroides	genus	−1.53	504.6	1475.0	0.018
Eisenbergiella	genus	−1.96	6.3	59.4	0.003
Hungatella	genus	−2.08	16.5	108.7	0.009
Burkholderiaceae	family	−2.14	11.6	92.1	<0.001
Ralstonia	genus	−2.17	11.2	92.1	<0.001
Frisingicoccus	genus	−3.99	0.2	182.1	0.003

LogFC is a binary logarithm of the ratio of the average proportion of bacteria of a given taxa in patients to that in healthy individuals.

2.2. Fecal Short-Chain Fatty Acids

Patients with I + D showed an increase in the total level of SCFAs as well as absolute levels of acetic acid, propionic acid, butyric acid, and isoacids in comparison with the control group (Figure 1). The IsoCn/Cn ratio increased and the anaerobic index decreased in these patients compared to those in healthy individuals. The relative levels of the main SCFAs and isoacids in the patient group did not differ significantly from those in the control group (Table 4).

Our study also included a correlation analysis between the absolute levels of SCFAs and the main bacterial taxa in the gut microbiota (Table 5).

The amount of acetate in feces correlated with the severity of diarrhea, defined as an increase in the frequency of stools (r = 0.364; p = 0.014) and loose stool (r = 0.377; p = 0.011), as well as with urgent need to have a bowel movement (r = 0.525; p < 0.001). The severity of the latter symptom also correlated with the amount of propionate (r = 0.459; p = 0.002), butyrate (r = 0.363; p = 0.014), and the total amount of SCFA (r = 0.434; p = 0.003) in the feces. The number of isoacids in the feces correlated significantly with the frequency of stools (r = 0.355; p = 0.017), but not with the severity of loose stools. The level of butyrate in feces correlated with the severity of flatulence (r = 0.339; p = 0.023). There were no significant correlations between fecal SCFA levels and other GSRS parameters, as well as with drugs used, and laboratory parameters.

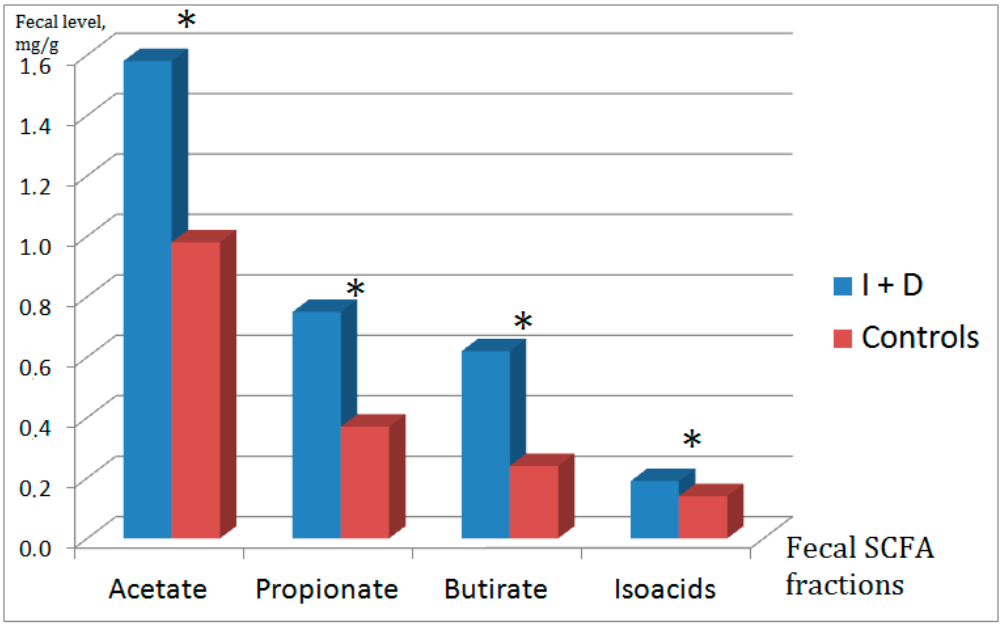

Figure 1. Absolute levels of fecal short-chain fatty acids (SCFA) fractions (acetate, propionate, butyrate, and isoacids) in diarrhea-predominant irritable bowel syndrome and functional dyspepsia combination (I + D) and in healthy individuals from the control group. *—the difference between the groups reached statistical significance.

Table 4. Absolute level of SCFAs (mg/g), IsoCn/Cn ratio, anaerobic index value, and relative level of SCFAs in patients with IBS-D and FD as well as healthy individuals in the control group.

Parameter	Group	Mean Value	SD	*p*
Absolute total level of SCFAs	I + D	3.19	1.69	0.003
	Control	1.81	1.29	
Absolute level of acetic acid (C2)	I + D	1.58	0.78	0.003
	Control	0.93	0.64	
Absolute level of propionic acid (C3)	I + D	0.75	0.49	0.002
	Control	0.37	0.3	
Absolute level of butyric acid (C4)	I + D	0.62	0.40	<0.001
	Control	0.24	0.21	
Absolute level of isoacids	I + D	0.19	0.10	0.028
	Control	0.14	0.1	
IsoCn/Cn	I + D	0.57	1.4	0.013
	Control	0.52	0.23	
Anaerobic index	I + D	−0.79	0.33	0.046
	Control	−0.69	0.19	
Relative level of acetic acid (C2)	I + D	0.55	0.07	0.111
	Control	0.56	0.12	
Relative level of propionic acid (C3)	I + D	0.24	0.05	0.463
	Control	0.23	0.04	
Relative level of butyric acid (C4)	I + D	0.21	0.06	0.178
	Control	0.17	0.07	
Relative level of isoacids	I + D	0.07	0.035	0.107
	Control	0.09	0.03	

Table 5. Significant correlations between the abundances of bacterial families in the gut microbiota and biomarkers of their metabolic activities (*r*; *p*).

Gut Microbiota Taxon	Acetic Acid (C2)	Propionic Acid (C3)	Butyric Acid (C4)	Isoacids	Total Acid Content	Anaerobic Index
Acidaminococcaceae			−0.55; <0.001			0.31; 0.04
Actinomycetaceae			0.30; 0.05	0.39; 0.008	0.30; 0.04	−0.45; 0.002
Aerococcaceae	0.42; 0.004	0.35; 0.02	0.55; <0.001		0.46; 0.001	
Akkermansiaceae				0.38; 0.01		0.32; 0.04
Anaerovoracaceae	−0.61; <0.001	−0.63; <0.001	−0.71; <0.001		−0.65; <0.001	0.51; <0.001
Atopobiaceae				0.39; 0.008		
Bacteroidaceae						0.39; 0.008
Barnesiellaceae		−0.35; 0.02	−0.49; <0.001		−0.36; 0.02	0.60; <0.001
Bifidobacteriaceae						−0.32; 0.03
Burkholderiaceae	−0.32; 0.03	−0.38; 0.01			−0.31; 0.04	
Butyricicoccaceae						0.37; 0.01
Carnobacteriaceae	0.33; 0.03	0.52; <0.001	0.47; 0.001		0.47; 0.001	−0.65; <0.001
Chitinophagaceae					−0.31; 0.04	0.32; 0.03
Christensenellaceae	−0.34; 0.02	−0.47; 0.001	−0.54; <0.001		−0.46; 0.001	0.57; <0.001
Clostridiaceae	−0.31; 0.04	−0.45; 0.002			−0.34; 0.02	0.35; 0.02
Comamonadaceae			−0.41; 0.005		−0.30; 0.05	0.42; 0.004
Defluviitaleaceae		−0.40; 0.007				
Enterococcaceae			0.51; <0.001		0.32; 0.03	−0.30; 0.04
Erysipelotrichaceae						−0.37; 0.01
Fusobacteriaceae		0.29; 0.05			0.31; 0.04	
Gemellaceae	0.46; 0.002	0.55; <0.001	0.63; <0.001		0.57; <0.001	−0.64; <0.001
Lachnospiraceae	0.32; 0.03	0.50; <0.001			0.32; 0.04	−0.55; <0.001
Lactobacillaceae	−0.35; 0.02				−0.30; 0.04	
Leuconostocaceae			−0.48; 0.0008			0.36; 0.02
Marinifilaceae	−0.37; 0.01	−0.41; 0.005	−0.39; 0.008		−0.33; 0.03	0.45; 0.002
Methanobacteriaceae	−0.48; 0.001	−0.42; 0.004	−0.45; 0.002		−0.40; 0.006	
Micrococcaceae		0.34; 0.02	0.38; 0.01		0.31; 0.04	−0.63; <0.001
Monoglobaceae						0.32; 0.03
Mycobacteriaceae	−0.32; 0.03	−0.39; 0.008	−0.34; 0.02		−0.33; 0.03	0.37; 0.01
Oscillospiraceae	−0.33; 0.03	−0.45; 0.002			−0.31; 0.04	0.34; 0.02
Oxalobacteraceae	−0.31; 0.04	−0.35; 0.02	−0.45; 0.002		−0.34; 0.02	0.43; 0.004
Pasteurellaceae		0.34; 0.02	0.57; <0.001		0.36; 0.01	−0.65; <0.001
Peptococcaceae	−0.33; 0.03	−0.33; 0.02				
Peptostreptococcaceae	−0.30; 0.05	−0.45; 0.002			−0.33; 0.03	0.32; 0.03
Puniceicoccaceae	−0.32; 0.03	−0.37; 0.01	−0.38; 0.01		−0.32; 0.03	0.40; 0.007
Rikenellaceae		−0.41; 0.005	−0.40; 0.007		−0.36; 0.01	0.56; <0.001
Saccharimonadaceae						−0.56; <0.001
Selenomonadaceae		0.33; 0.02	0.41; 0.005	−0.30; 0.05		−0.50; <0.001
Solimonadaceae	−0.38; 0.009	−0.40; 0.007	−0.34; 0.02		−0.36; 0.01	
Solirubrobacteraceae	−0.30; 0.05	−0.34; 0.02	−0.32; 0.03		−0.31; 0.04	0.33; 0.03
Streptococcaceae		0.37; 0.01	0.42; 0.004		0.33; 0.03	−0.74; <0.001
Sutterellaceae						0.33; 0.03
Tannerellaceae		−0.31; 0.04	−0.44; 0.002		−0.32; 0.03	0.55; <0.001
Vibrionaceae			−0.31; 0.04			
Weeksellaceae	−0.30; 0.05	−0.34; 0.02	−0.32; 0.03		−0.31; 0.04	0.33; 0.03

3. Materials and Methods

3.1. Patients

This study was approved by the Local Ethic Committee (No. 06-21 d/d 7 April 2021) of Sechenov University, and all participants provided written informed consent. All patients admitted to the Department of Chronic Intestinal and Pancreatic Diseases of the Clinic with complaints of loose stools and dyspepsia were screened for inclusion. The inclusion criterion was the presence of both IBS-D and FD. The clinical diagnosis was established

based on the Rome IV Criteria [1,2] and the exclusion of organic diseases according to the results of an examination as recommended the national guidelines [3,13].

The exclusion criteria were as follows: presence of digestive disease other than IBS and FD (including Helicobacter pylori infection, inflammatory bowel diseases, microscopic colitis, and celiac disease); age under 18 years and over 59 years; refusal to sign the informed consent form for participation in this study; pregnancy; lactation; history of cancer or digestive tract surgeries (except for appendectomy); renal failure (creatinine clearance < 50 mL/min); liver failure, confirmed by clinical and laboratory data; other severe somatic diseases (any disease or conditions threatening the patient's life or worsening the prognosis); known sensitivity to any component of the drugs to be administered in this study; a positive blood test result for biomarkers of human immunodeficiency virus infection, syphilis, hepatitis B or/and C; and use of drugs that could affect the composition of the gut microbiota (probiotics, prebiotics, antibiotics, prokinetics, and proton pump inhibitors) within the previous three months. The stool of the patients was subjected to microbiological analysis. When intestinal pathogens (Salmonella, Shigella and others) were detected, such patients were excluded as having organic bowel disease.

In total, 60 of 350 screened patients met the criteria described above and were enrolled in this study. The control group consisted of 20 healthy individuals aged 18–59 years, comparable to the patient group in sex and age, without gastrointestinal tract complaints and concomitant diseases of the respiratory, urinary, endocrine, and cardiovascular systems, who applied to the clinic for scheduled prophylactic medical examinations. The study design is shown in Figure 2.

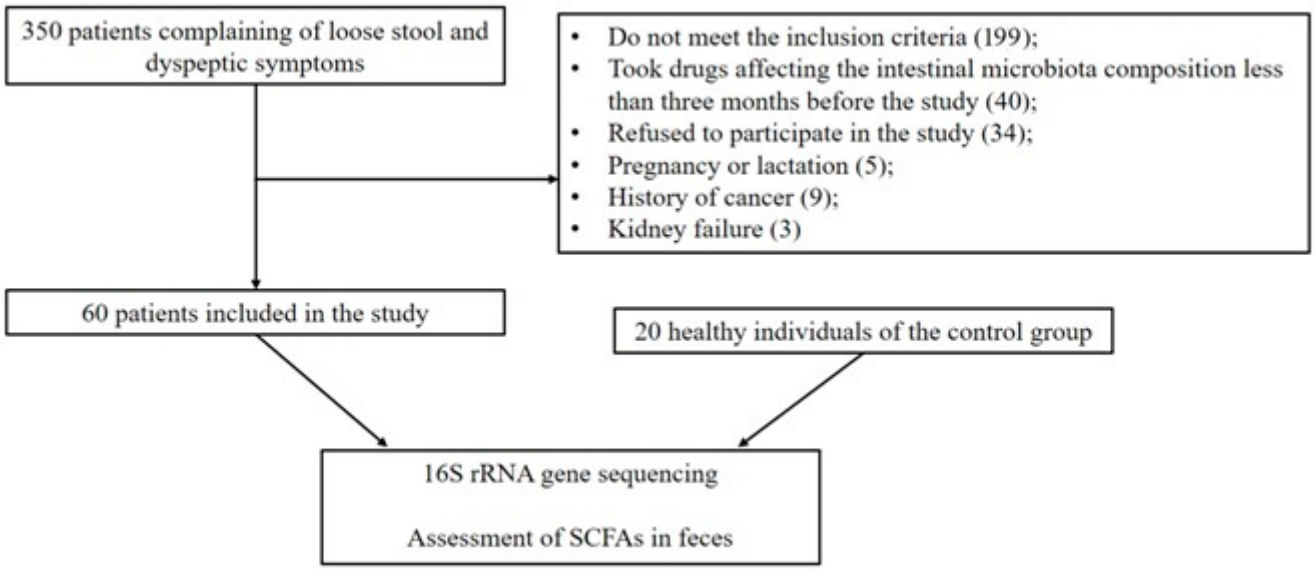

Figure 2. Flow diagram.

3.2. Severity of Symptoms Assessment

The severity of symptoms was assessed using the Gastrointestinal Symptom Rating Scale (GSRS) [14]. Symptoms were scored semiquantitatively from 1 (no symptom) to 7 (very severe symptom).

3.3. 16S rRNA Genes Library Preparartion and Sequencing

After receipt from the patients, the samples were frozen and stored at −70 °C until sample preparation. Patient and control samples were processed and sequenced in parallel. Just before the library preparation, the frozen samples were placed in a container with ice to thaw for 30 min. A 10 µg sample was taken with a spatula and placed in test tubes for homogenization. Sample tubes were incubated for 10 min at 65 °C, and then for 10 min more at 95 °C. Subsequently, the samples were homogenized using a MagNA

Lyser automatic homogenizer (Roche, Basel, Switzerland) according to the manufacturer's instructions, following which they were centrifuged at 14,000 rpm for 10 min. The resulting supernatant (400 µL) was used for further isolation of nucleic acids. Total DNA was isolated using reagents of the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland) in a MagNA Pure LC automated nucleic acid extraction system. The isolated DNA was stored at −20 °C. A NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for DNA qualitative and quantitative evaluation. The 16S metagenomic libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA) recommended by Illumina for the MiSeq sequencer. The following primers were used for the amplification of V3-V4 16S rDNA variable regions: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC [15]. The part of the sequence before the dash refers to Illumina adapters. These primers are aimed at the amplification of bacterial (more than 90% taxon coverage) but not archaeal (less than 5%) rRNA genes. The average amplicon length was approximately 450 bp with minimal variation. Applied Biosystems 2720 Thermal Cycler amplifier (Thermo Fisher Scientific, Waltham, MA, USA) was used.

The amplification program was as follows: 95 °C—3 min; 30 cycles: 95 °C—30 s, 55 °C—30 s, 72 °C—30 s; 72 °C—5 min; 4 °C finally. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Blair, CA, USA) according to the manufacturer's protocol.

Then, the second round of amplification was performed for double indexing of samples using a specific combination of index sequences from the Nextera XT Index kit (Illumina, San Diego, CA, USA). The amplification program was similar except that the number of cycles was 8. PCR products were also purified using Agencourt AMPure XP beads. The concentration of the resulting 16S libraries was determined using the Qubit® 2.0 fluorimeter (Invitrogen, Waltham, MA, USA) and QuantiT™ dsDNA High-Sensitivity Assay Kit.

The purified amplicons were mixed equimolarly according to the obtained concentrations. The quality of the prepared libraries was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent DNA 1000 Kit Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Sequencing was performed with the MiSeq sequencer (Illumina, San Diego, CA, USA) in the paired-end mode (2 × 250 bp) using the MiSeq Reagent Kit v2.

3.4. 16S rRNA Sequencing Data Analysis

First, forward and reverse Illumina reads were pre-trimmed with Trimmomatic 0.38 and merged with MeFiT tool [16] into a single amplicon sequence sequence (because of the small length of the overlapping regions with high quality). Then, the merged reads were processed with the DADA2 1.22 package (Bioconductor project) [17], including reads filtering, denoising, RSV inference and detecting chimeras. Taxonomic annotation was performed using the naive RDP classifier algorithm (built-in default DADA2 annotation engine) [18] based on the Silva 138.1 database [19]. The taxon assignment confidence threshold was set to 80%. It should be noted that the reads were merged even before the DADA2 analysis because of the relatively small overlap region size.

To take into account the compositionality of microbiome communities, the differential taxon abundance analysis was performed using the ALDEx2 package [20] (Bioconductor) for centered log ratio (CLR)-transformed read counts. The default Monte-Carlo samples count was elevated to 1000. We considered the Mann–Whitney test for CLR values as the main criterion, due to the high heterogeneity of the patients' samples. Welch's *t*-test and GLM/ANOVA were also applied for CLR values. Additionally, we performed the same tests for normalized reads counts without CLR transformation. Finally, the false discovery rate (FDR) calculation was performed with the Benjamini–Hochberg algorithm.

3.5. Evaluation of the Content of Acetic, Propionic, Butyric, Acids and SCFA in Fecal Samples

In all study participants, the absolute and relative content of acetic (C2), propionic (C3) and butyric (C4) acids, the level of isoacids (SCFA isomers), and the ratio of isoacids to unbrached acids (isoCn/Cn) were determined with the following method. Fecal samples were collected from all study participants and kept at −80 °C until further analysis. After defrosting, 0.1 g fecal sample was placed in a tube with a conical bottom, and 2 mL distilled water and 1 mL calibration solution was added and mixed by shaking for 10 min. Further, 0.5 mL of 1 N HCl was added and the mixture was centrifuged at 5000 rpm for 10 min. A volume of 1 µL supernatant was injected with a microsyringe into the Khromos GCh-1000 gas chromatograph evaporator with a flame ionization detector equipped with a 36 m-long quartz capillary column with an inner diameter of 0.32 mm and with a stationary free fatty acids phase in the form of films 0.33 µm thick. The chromatograph operation mode was isothermal with a thermostat temperature of 150 °C, and an evaporator and detector temperature of 230 °C. The carrier gas was nitrogen, with a column inlet pressure of 1.8 atm. Carrier gas flow was 2.0 mL/min, and air flow was 300 mL/min. Chromatography time was approximately 8 min. Determination of the absolute content of individual acids in a mixture is carried out by calculating the areas of chromatographic peaks both by the "triangle" method and by computer processing of chromatograms.

3.6. Statistical Analysis

Statistical data processing was performed using STATISTICA 10 software (StatSoft Inc., Tulsa, OK, USA). Differences between groups in continuous variable values were assessed using Student's *t*-test for normal distribution and the Mann–Whitney method for other distributions. Comparison of the abundance of gut microbiota taxa was carried out using the Mann–Whitney method corrected for FDR (see above). The abundance of gut microbiota taxa are presented as the average number of reads in the studied groups. Significant differences in categorical variables were determined using Fisher's exact test. Spearman's correlation was used to assess the links between the abundance of the taxa and SCFA levels. If the probability of making a type I error was $p < 0.05$, the difference was considered significant.

4. Discussion

When studying the composition of the gut microbiota in patients with IBS, different data have been obtained in various studies. According to two meta-analyses, individuals with IBS demonstrate a decrease in the levels of *Lactobacillus* (phylum Firmicutes) and *Bifidobacterium* (phylum Actinobacteria) in feces [21,22]. A meta-analysis of 16 studies performed by Duan et al. showed an increase in the abundance of Firmicutes in the gut microbiota of patients with IBS; this was mainly due to an increase in the number of bacteria belonging to order Clostridiales [23]. Most recent meta-analysis revealed that Bacteroides, *Fecalibacterium prausnitzii*, *Ruminococcus* spp., and *Bifidobacteria* implicated in IBS and treatment response [10]. However, in these cases, the microbiota of patients with IBS was analyzed without considering the clinical variants of the disease course.

Some changes in the gut microbiota composition have been observed in IBS-D. According to Zhuang et al., these patients with IBS-D showed a decrease in the abundance of Firmicutes and an increase in the abundance of the Bacteroidetes in feces, when compared to healthy individuals [24]. Among Firmicutes, at the family level, the abundance of Ruminococcaceae and Lachnospiraceae reduced [25] and at the genus level, according to Su et al., the abundance of *Lactobacillus* was reduced [26]. Liu et al. reported a decrease in the abundance of *Fecalibacterium prausnitzii* (phylum Firmicutes) in the feces of patients with IBS-D [22]. In addition, according to the results of Rangel et al., the abundance of bacteria belonging to phylum Firmicutes in IBS-D patients exceeds that in healthy individuals, including the classes Bacilli and Clostridia [26]. According to Carroll et al., patients with IBS-D have an increased abundance of Proteobacteria due to an increased in abundance of Enterobacteriaceae [27].

Data on changes in Bacteroidetes levels are contradictory. According to Krogius-Kurikka et al., patients with IBS-D have a reduced number of bacteria of this phylum when compared to healthy controls; however, according to Shukla et al., the level of bacteria of the Bacteroides genus in the feces of patients with IBS-D is higher than that in healthy individuals [28,29].

In the current study, patients with IBS-D and FD overlap also had increased abundance of bacteria belonging to phylum Firmicutes and decreased Bacteroidetes level in the gut microbiota in comparison with healthy controls. However, after adjusting for FDR, these differences were no longer significant. At the same time, there continued to be a significant increase in the abundance of representatives of Erysipelotrichales and Lachnospiraceae, which belong to phylum Firmicutes. Lachnospiraceae included Clostridium cluster XIVa that are involved in immune response regulation due to an increase in T-regulatory lymphocyte (Treg) production. A decrease in the Treg pool can lead to the development of inflammatory changes in the intestinal wall, and Treg synthesis induction, in turn, helps reduce the severity of this reaction [30]. Since IBS is characterized by a permeability disorder of the intestinal barrier, an increase in the levels in Lachnospiraceae may prevent the pronounced development of inflammation in the intestinal wall in response to bacterial translocation. Moreover, previous studies established a direct correlation between the content of Erysipelotrichales class bacteria and expression of tumor necrosis factor alpha (TNF-α) [31] and also showed an increase in the expression of TNF-α in the mucous membrane of the small and large intestine in patients with IBS [32,33].

Changes in the gut microbiota composition in patients with functional gastrointestinal diseases affect the normal activity of the gastrointestinal tract owing to the impact on motility, sensitivity, permeability of the mucoepithelial barrier, and regulation of the local immune response [34,35] which is related to a change in the level of SCFAs.

The ratio of acetate, propionate, and butyrate has a normal value close to 60:20:20 [36]. The concentration of isoacids (isobutyric, isovaleric, and isocaproic) makes it possible to evaluate proteolytic bacterial activity as the ratio of all isoacids to unbranched chain acids (IsoCn/Cn) [9]. The anaerobic index reflects the ratio of aerobic to anaerobic bacteria. The shift of this index towards more negative values indicates a change in the balance of aerobic and anaerobic microorganisms in favor of the latter [9].

The results of a meta-analysis of 15 studies conducted by Sun et al. suggest that patients with IBS-D have an increased concentration of butyric acid in fecal samples [11]. Farup et al. demonstrated that patients with IBS-D, as compared with the control group, showed an increased content of acetate and propionate as well as an increase in the total concentration of SCFAs in their feces in comparison with healthy controls [12].

In our study, patients with IBS-D and FD overlap demonstrated an increase in the absolute levels of the main types of SCFAs: acetate, propionate, and butyrate. The observed increase in absolute propionic acid content is consistent with the results of almost all previously published studies where patients with IBS-D were examined as the main group. The data on changes in the content of acetic and butyric acids are contradictory; studies have reported both a decrease [37] and an increase in the level of these acids [38] as well as a level comparable to that of the control [39].

SCFAs are involved in several physiological processes. Their regulatory functions depend on the activation of specific G protein-coupled receptors, which subsequently initiate cell-specific signaling cascades. Butyric acid is known to inhibit interleukin-12 and increases the production of interleukin-10, thereby preventing the formation of an excessive immune response [12]. However, butyric acid enhances gastrointestinal motility, which can lead to diarrhea. In IBS-D, the first effect is beneficial, considering the regression of inflammatory changes in the intestinal wall, while the second mechanism may augment clinical manifestations. Although in our study it was butyrate that did not show a significant correlation with the severity of diarrhea, it did exist for acetate and isoacids. At the same time, almost all SCFA fractions showed a positive correlation with the severity of the need to urgently empty the intestines. This confirms the controversial role of SCFA in IBS-D.

Butyric acid also contributes to maintaining favorable conditions for bacterial activity by limiting oxygen diffusion from colonocytes to the luminal part of the colon [40].

Acetic and propionic acids, which are ligands of the GPR43 receptor, inhibit the NF-κB signaling pathway and reduce the local inflammatory response in the intestine [41,42]. In addition, propionic acid interacts with the GPR41 receptor and reduces the secretion of pro-inflammatory cytokines, such as interleukins-4 and -5, and TNF-α [43].

Indirect proof of the beneficial effect of increased SCFA production in IBS-D on the suppression of intestinal inflammation can be found when analyzing changes in the production of these acids in inflammatory bowel disorder (IBD). A meta-analysis from Xu et al. showed a decrease in the content of all main forms of SCFAs (acetic, propionic, and butyric acid) in the exacerbation of ulcerative colitis when compared to that in healthy individuals. Concurrently, the level of butyric acid increased, compared to that in the control group, in patients with ulcerative colitis in remission [44]. Therefore, marked inflammation in the intestine during IBD exacerbation is associated with a low level of SCFAs in the feces, while minimal inflammation in IBS and IBD remission is associated with their high levels. This suggests that the metabolic function of the intestinal microbiota may play an important role in both the development of IBD remission and in maintaining intestinal inflammation at a minimum level in IBS, preventing its potential transformation into IBD.

Normally, SCFAs are synthesized in a certain proportion, close to 60:20:20. A change in the relative content of the main SCFAs may indicate the predominance of the metabolic activity of aerobic or anaerobic flora [9]. However, no data suggesting a significant change in the relative content of SCFAs were obtained in this study. When analyzing the literature data, we did not find information on whether this proportion was maintained in patients with IBD.

SCFA isoforms are normally formed in minimal amounts in the intestines. Intestinal mucus proteins normally serve as substrates for the formation of isoSCFAs. Therefore, the increase in the number of isoacids and the ratio of IsoCn/Cn observed in our patients suggests an increase in the activity of bacteria with a pronounced proteolytic potential. According to the literature, pronounced proteolytic activity is typical for *Streptococcus*, Lachnospiraceae, Lactobacillales, *Clostridium*, and Pseudomonadaceae [45]. In our study, patients had a significantly higher content of bacteria of the Pseudomonadaceae family, compared to the control group, which may be the reason for the increase in the overall proteolytic activity of the microbiota.

A limitation of our study is the lack of analysis of intestinal inflammation markers, including fecal calprotectin levels. Further research is required to verify our finding in larger and different populations and to clarify the role of the intestinal microbiota in inhibiting the progression of minimal intestinal inflammation in functional digestive diseases.

5. Conclusions

This study revealed that patients with IBS-D and FD overlap demonstrated changes in the composition and function of the gut microbiota that can favor the inhibition of intestinal inflammation in response to bacterial translocation caused by an increase in the permeability of the intestinal barrier in IBS.

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