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RESEARCH OF THE SPECIES COMPOSITION OF THE INTESTINAL MICROBIOM WITH THE USE OF TARGET SEQUENCY GENERATION 16S pRNA

Abstract. The intestinal microbiome is an extracorporeal organ and is characterized by a complex hierarchical structure that performs a variety of functions, depending on its quantitative and species composition. Violation of its balance can lead to various pathological conditions (atherosclerosis, diseases of the cardiovascular system, inflammatory bowel disease, type 2 diabetes mellitus, etc.).

In order to study the species ratio of bacteria in the intestine, the characteristics of bacterial species and enterotypes, a molecular genetic study of the 16S rRNA gene of microorganisms was carried out using the next generation technology (NGS).

Using the semiconductor sequencing method, the first results of the analysis of the species composition of the intestinal microbiome of 33 patients were obtained. Five main phyla were identified: Actinobacteria (4.9%), Bacteroidetes (37%), Firmicutes (42%), Proteobacteria (16%) and Vericomicrobia (0.02%). Found 298 basic taxonomic units.

The analysis showed that bacteria are present in small numbers in the bacterial population, which are indicators of a healthier metabolic status of the intestine.

When phylogenetic analysis of the bacterial population was carried out, 2 enterotypes were identified: in 58% of patients, 1 enterotype of Bacteroides, in 42% of patients 2 enterotype of Prevotella.

Metagenomic analysis of the species ratio of bacteria showed that a decrease in the species diversity of bacteria was observed in the intestine. There is a decrease in bacteria responsible for immunity, a high proportion of bacteria predisposing to the development of type 2 diabetes mellitus, metabolic syndrome and inflammatory bowel diseases.

Keywords: intestinal microbiome, 16S rRNA gene, gut microbiota.

The development of next-generation sequencing technologies has significantly improved our understanding of the species ratio of the bacterial population in the human intestine.

Actively carried out work on the identification, description and quantification of bacterial populations of the gastrointestinal tract, the relationship between the species ratio of bacteria and the prevalence of diseases. The knowledge gained will allow the development of new diagnostic and prognostic therapeutic strategies.

Intestinal microbiome is a unique organ, the formation of which begins from the 24th week of pregnancy from the mother with single colonies of Escherichia coli and lactobacilli [1]. For a long time, it believed that colonization of the gastrointestinal tract by microorganisms occurs after birth. However, recent studies have shown that bacteria are present in the placenta, amniotic fluid, umbilical cord blood, meconium [1, 2, 3]. The composition of the intestinal microbiome depends on the environment and delivery methods and on breastfeeding [4-6].

It is believed that the final formation of the enterotype of the microbiome begins at 18 months [4, 5, 7].

By about 2-3 years, the microflora undergoes the latest changes, an "adult" microbiota is formed, 60-70% of which will slightly change throughout life [3,7,8].

Improper nutrition of the mother during pregnancy or in a child in early childhood can lead to depletion and a defect in the gut microbiota.

A microbiome of the human intestine consists of approximately 1.5 kg of cells, most of which are bacterial, and a minority belongs to archaea and eukaryotes. In western populations, phyla Bacteroidetes and Firmicutes, as a rule, dominate the intestines, while other phyla make up 10% or less [14].

The microbiome of the human intestine consists of approximately 1.5 kg of cells, most of which are bacterial, and the minority belongs to archaea and eukaryotes, contains up to 1000 species of bacteria encoding about 5 million genes. In the normal microbiota, both commensals and opportunistic microorganisms are found.

Microbiota is necessary for the proper growth of the body, the development of immunity and nutrition. Microorganism communities are in close interaction with our body, performing many functions necessary for the physiology and survival of the body [5,9,10,11]. Disorders of microbiome homeostasis play an important role in the development of inflammatory bowel diseases, atherosclerosis, obesity, metabolic syndrome (MS), type 2 diabetes mellitus (DM) [2,6,7,9].

The aim of our study: to study the species composition of the intestinal microbiome using targeted semiconductor sequencing of the 16s pPHK gene.

Materials and methods. OBJECT OF STUDY. The study involved 33 patients sent by a gastroenterologist to study the species composition of the microbiome by the method of targeted semiconductor sequencing of the 16SrRNA gene of microorganisms using the next generation technology (NGS).

Total DNA was extracted from 250.0 mg of a homogenized wet fecal sample using the PureLinkTM Microbiome DNA Purification Kit (TermoFisher Scientific, USA) DNA isolation kit according to the manufacturer's instructions. Sequencing of the 16S rRNA gene was carried out on a new-generation semiconductor sequencer Ion Gene Studio S5 Plus (TermoFisher Scientific, USA) in the laboratory of personalized genomic diagnosis of the "Medical Centre Hospital of the President's Affairs Administration of the Republic of Kazakhstan".

DNA libraries (the set of nucleotide sequences of the genomic DNA of the studied samples) were prepared in accordance with the Ion 16STM Metagenomics Kit protocol (Termofisher Scientific, USA).

The preparation of the library took place in several stages:

1. Obtaining a PCR product by amplification of the hypervariable region 16S, followed by purification and measuring the concentration of the PCR product.
2. Directly preparing the library by ligation with barcoding, cleaning the ligated adapter library.
3. The concentration of the obtained library was measured on a QS 12K Flex analyzer.

The concentration of DNA libraries was determined on a QuantStudioTM 12K Flex system using the Ion Library TaqMan® Quantitation Kit (Termofisher Scientific, USA). Figure 1 shows an example of measuring library concentration.

The sequence of amplified fragments was carried out in the Ion PGM ™ system, bioinformatics analysis of the results was carried out using the Ion Reporter ™ software, the Ion 16S ™ Metagenomics Kit analysis module.

Using a combination of two primer pools allows a wide range of bacteria in a mixed population to be identified by sequence of bases. Figures 2 and 3 show examples of bioinformatics analysis and a Crohn's diagram.

Results. Metagenomic analysis showed that the fecal mass consist of5 main phyla: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Vericomicrobia were found in a bacterial population. The proportion of each phyla in the total bacterial population is different. The smallest amount falls on Phyl Vericomicrobia (0.02%), the main filaments are Bacteroidetes (37%) and Firmicutes (42%). The results are presented in table.

A total of 298 bacterial species were identified in 33 patients in the intestinal microbiome. Of these, 79% of the bacterial population is represented by 38 to 82 species. In 21% (7 patients), intestinal microbiomes are represented by a small number of bacteria (from 20 to 30 species).

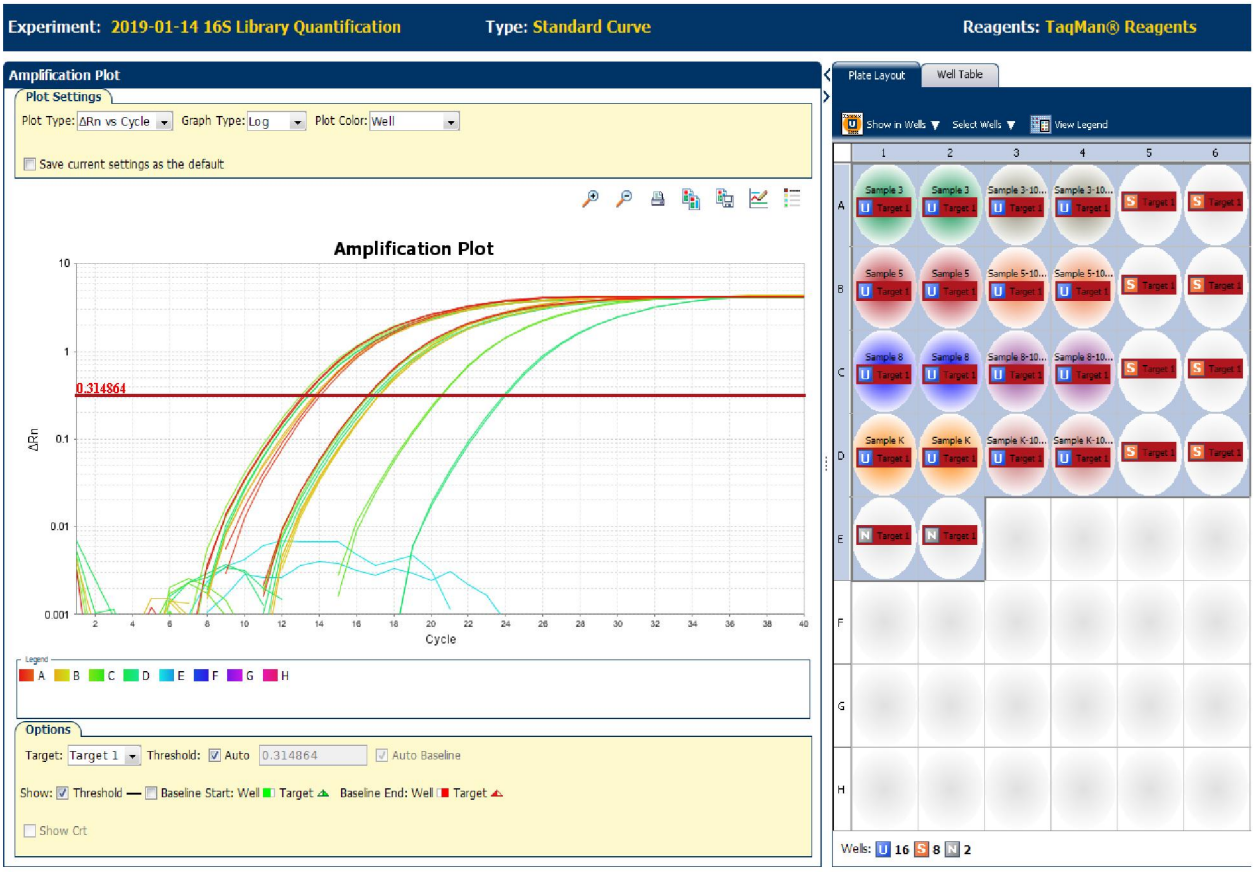

Figure 1 – Melting curves of samples in dilutions of 1: 100 and 1: 1000 relative to standards when measuring library concentration

Phylum	Class	Order	Family	Genus	Species	% ID	Count	DB counters	F:R %	% of total reads	% of valid reads	% of mapped reads	% of mapped reads per primer
							32474	**8761 : 23713**		**22.32**	**43.27**	**100**	**100**
Actinobacteria							335	216 : 119		0.23	0.45	1.03	1.03
	Actinobacteria						251	216 : 35		0.17	0.33	0.77	0.77
		Bifidobacteriales					97	81 : 16		0.07	0.13	0.3	0.3
			Bifidobacteriaceae				97	81 : 16		0.07	0.13	0.3	0.3
				Bifidobacterium			97	81 : 16		0.07	0.13	0.3	0.3
					(genus level ID only)		16	0 : 16		0.01	0.02	0.05	0.05
					(slash calls)		12	12 : 0		0.01	0.02	0.04	0.04
					catenulatum	99.55 - 99.55	50	50 : 0	0 : 100	0.03	0.07	0.15	0.15
					ruminantium	100 - 100	19	19 : 0	0 : 100	0.01	0.03	0.06	0.06
		Coriobacteriales					154	135 : 19		0.11	0.21	0.47	0.47
			Coriobacteriaceae				154	135 : 19		0.11	0.21	0.47	0.47
				(family level ID only)			19	0 : 19		0.01	0.03	0.06	0.06
				(slash calls)			33	33 : 0		0.02	0.04	0.1	0.1
				Collinsella			102	102 : 0		0.07	0.14	0.31	0.31
					aerofaciens	99.35 - 100	102	102 : 0	100 : 0	0.07	0.14	0.31	0.31

Figure 2 – An example of the result of bioinformatic analysis of a microbiome based on Ion Reporter software

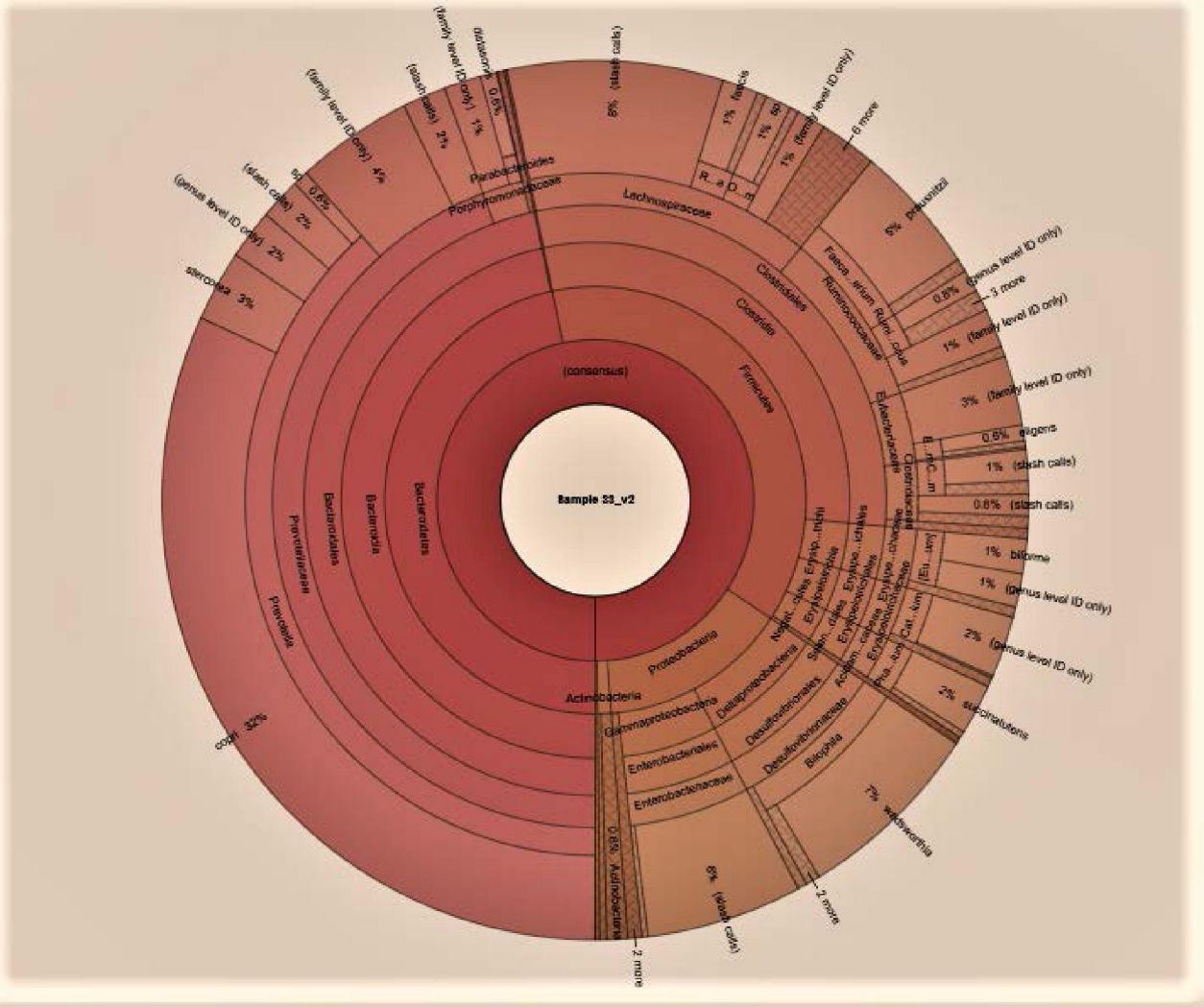

Figure 3 – Phylogenetic metagenomic classification of taxonomic units (Crohn's diagram)

The ratio of bacterial species in the intestinal microbiome

Phyla	Class	Order	Family	Genus	Species	Share, %
Actinobacteria	1	3	3	13	34	4,9
Bacteroidetes	1	1	4	10	46	37
Firmicutes	4	5	9	52	164	42
Proteobacteria	4	4	4	17	29	16
Vericomicrobia	1	1	1	1	1	0,02

The proportion of Actinobacteria phyla is 4.9%, but the structure is heterogeneous. Only 19% of patients in the bacterial population identify three taxonomic groups Actinomycetales, Bifidobacteriales and Coriobacteriales, in 70% of the patients the bacteria Bifidobacteriales and Coriobacteriales are present in the microbiome, and in 11% of the patients, the Actinobacteria class is represented only by the bacteria of the Coriobacteriales order.

Phylogenetic analysis revealed that in the bacterial population, Bifidobacteriales were found in the microbiota in 85% of patients. However, their number in the structure is heterogeneous, a wide range is observed from 0.01 to 16.62%, only in the microbiome of 4 patients their number was from 8% to 16%.

Phyla Bacteroidetes werefound in 31 patients. Cluster analysis of the phyla showed that in the bacterial population it accounts for 37%. Phyla is represented by 10 genus and 20 species of bacteria. The

main taxonometric groups Bacteroides / Odoribacter / Parabacteroides / Prevotella / Alistipes have been identified.

When carrying out a phylogenetic analysis of the bacterial population of 33 patients, 2 enterotypes were identified: in 58% of patients - Bacteroides, in 42% - Prevotella.

One of the main commensal bacteria in the gut microbiome is Phyl Firmicutes [8, 12, 13]. So in the bacterial population, intestinal microbiome phyla was diagnosed in 33 patients and makes up 42% of the entire bacterial population. Phylogenetic analysis of the Firmicutes phyla showed that it consists of 4 classes, 5 orders, 9 families, 52 genus, and 164 species.

A metagenomic analysis of the structure of the Proteobacteria phyla showed that it consists of 4: classes, 4 orders, 4 families, 17 genera, and 29 species. Phyla Proteobacteria occupies 16% of the entire bacterial population of the studied individuals.

The fifth phyl Vericomicrobia is represented by one bacterial species, Akkermansia muciniphila. This type of bacteria in the intestinal microbiome was found in only 8 patients. The phyla share in the total bacterial population is 0.02%.

Discussion. According to European researchers, intestinal microbiomes mainly consist of bacterial types such as Firmicutes (60–65%), Bacteroidetes (20–25%), Proteobacteria (5–10%) and Actinobacteria (3%) [1, 14, 15]. The ratio of phyla is greatly influenced by the place of residence, food culture. Active work is underway to study the general population of bacteria in the human intestine. Several groups have studied the commonality of intestinal microbiome. One of the first observations was that the human intestinal flora mainly belongs to only two types of Firmicutes (mainly represented by Clostridia) and Bacteroidetes, with fewer bacteria belonging to Proteobacteria and Actinobacteria [1, 16].

The data obtained on the species composition of the bacterial population of the Actinobacteria phyla show a decrease in bio-bacterial species in the intestine, which is associated with the occurrence of gastrointestinal tract disorders, and in 81% of patients there is a violation of intestinal homeostasis, an imbalance in the microsystem, which leads to the onset of the disease.

In the structure of phyla Propionibacteria, Rothia, which are indicators of a healthier metabolic status of the intestine, were detected in the structure of the phyla not detected, i.e., an analysis of the structure showed a decrease in the species diversity of bacteria.

Phyla Bacteroidetes, the predominant genus in the human intestine, plays an important role in numerous metabolic processes and can provide a certain level of protection against the multiplication of pathogenic microbes in the intestine. Bacteria are involved in the process of fermentation of carbohydrates, protein utilization, and biotransformation of bile acids. Actively decompose carbohydrates, contribute to the production of vitamins C, B2, B5, H. [2, 15].

According to the composition of the bacterial population of Phyla Bacteroidetes, the researchers proposed to identify certain enterotypes [18] regardless of location, health status or age. Three enterotypes are distinguished in the bacterial population by the dominance of representatives of the genus: Bacteroides, Prevotella, Ruminococcus. However, there are no clear criteria for determining enterotypes [17, 18].

Bacteroides enterotype bacteria are characterized by activity against the decomposition of carbohydrates, contribute to the production of vitamins C, B2, B5, H. It is assumed that patients with this enterotype will be less likely to suffer from atherosclerosis or it will manifest at a later date. The Bacteroides enterotype is typical for people who eat large amounts of meat and animal fats[13].

Enterotype - Prevotella, bacteria in the intestines do not process sugar and carbohydrates well, provide the body with vitamin B1 and folic acid. But with this enterotype, the load on the intestinal mucosa can increase. The risk of obesity and the development of metabolic syndrome, cardiovascular disease are characteristic. In the process of life, bacteria are able to destroy the protective mucous membrane, predisposing to defects in the intestinal mucosa, ulcerative colitis and inflammatory bowel diseases [19, 20].

Enterotype - Ruminococcus. These bacteria increase the absorption of carbohydrates, as well as blood sugar. Representatives of this enterotype synthesize folic acid and vitamin B1. This enterotype is most common in countries where plant foods predominate in the diet.

The obtained data on the species composition of the bacterial Bacteroidetes phyla population in the intestine showed that 2 enterotypes, Bacteroides, Prevotella, were isolated. Probably, the prevalence of two enterotypes is associated with the peculiarities of Kazakh nutrition, the predominance of meat and

fatty foods in the diet. The presence of a large percentage of Prevotella bacteria indicates a predisposition of patients to inflammatory processes in the intestine (Crohn's disease, ULC).

An increase in the population of Firmicutes phyla bacteria contributes to an increase in body weight, the onset of type 2 diabetes mellitus, cardiovascular pathology and inflammatory bowel diseases. Patients with a predominant bacterial population of Firmicutes are more likely to suffer from atherosclerosis or this pathology manifests itself at an earlier age [20].

It is known that an increase in the number of Firmicutes representatives and a decrease in the number of Bacteroidetes leads to the development of a metabolic syndrome [18]. When studying the Bacteroidetes / Firmicutes ratio in 63.6% of patients, the taxonomic groups of Firmicutes phyla bacteria prevail in the bacterial structure.

The obtained data on the species ratio of Firmicutes phyla show that patients are predisposed to the development of obesity and insulin resistance, i.e. Type 2 diabetes. The prevalence of Firmicutes phyla bacteria can be explained by the predominance of a high-fat diet in nutrition, which leads to inflammation of the intestinal mucosa and to a decrease in lactobacilli.

Phyla Proteobacteria, the most numerous group of bacteria (1534 species are described), is a heterogeneous group, this group includes both symbionts of eukaryotes and a large number of pathogenic and conditionally pathogenic microorganisms [21].

Phyla Proteobacteria is divided into five classes: alpha, beta, delta, gamma and epsilon proteobacteria. When analyzing the species composition of the intestinal microbiome of 33 patients, it was found that there are 4 classes of bacteria in the microbiota: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, no Epsilon proteobacteria class was found in any patient. Pathogenic bacteria were found in a number of patients, Haemophilus parainfluenzae, 18% Echerichia coli were found in 40%, Klebsiella pneumonia in 12%, and Mannheimia varigena in 13%.

The results of the phylogenetic analysis of the Proteobacteria phyla show that there is a decrease in the species diversity of bacteria, but at the same time, metagenomic analysis allows the detection of pathogenic bacteria using accessible methods that are difficult to find.

The bacterium Akkermansia muciniphila, a filial representative of Vericomicrobia, is considered an important indicator of metabolic intestinal health [12,22]. Bacteria should be widely present in the human intestine, their share should be 1-4% of the bacterial population in the colon. Akkermansia muciniphila is a mucin-degrading bacterium that produces short-chain fatty acids that have beneficial effects on intestinal cells and immunity[12,18,22]. A low indicator of the presence of bacteria in the colon in the studied patients indicates inflammatory processes in the intestine, probably due to the peculiarities of the nutrition of the mother and child in the first 18 months of life, the culture of nutrition.

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16S рРНК ГЕНІҢІҢ ТАРГЕТТІК СЕКВЕНДЕУІН ПАЙДАЛАНА ОТЫРЫП ІШЕК МИКРОБИОМЫНЫҢ ТҮР ҚҰРАМЫН ЗЕРТТЕУ

Аннотация. Ішек микробиомы экстракорпоральдық органға жатады және оның сандық және түрлік құрамына байланысты әртүрлі функцияларды орындайтын күрделі иерархиялық құрылыммен сипатталады. Оның балансының бұзылуы әртүрлі патологиялық жағдайлардың туындауына әкелуі мүмкін (атеросклероз, жүрек-қантамыр жүйесінің аурулары, ішектің қабыну аурулары, 2 типті қант диабеті және т.б.).

Ішектегі бактериялардың түрлік ара қатынасын, бактериялық түрлер мен энтеротиптердің ерекшеліктерін зерттеу мақсатында келесі буын технологиясын (NGS) қолдана отырып, микроорганизмдердің РНК 16s генін молекулалық-генетикалық зерттеу жүргізілді.

Жартылай өткізгіш секвендеу әдісін пайдалана отырып, 33 пациенттің ішек микробиомының түрлік құрамын талдаудың алғашқы нәтижелері алынды. 5 негізгі фил сәйкестендірілді: Actinobacteria (4,9%), Bacteroidetes (37%), Firmicutes (42%), Proteobacteria (16%) және Vericomicrobia (0,02%). 298 негізгі таксономиялық бірлік табылды.

Талдау бактериялар популяцияда ішек метаболизмінің неғұрлым сау статусының индикаторлары болып табылатын бактериялар аз мөлшерде көрсетілгенін көрсетті.

Бактериялық популяцияның филогенетикалық талдауын жүргізу кезінде 2 энтеротип анықталды: 58% пациентте -1 Bacteroides энтеротипі, 42% - 2 Prevotella энтеротипі.

Бактериялардың түрлік ара қатынасын метагеномдық талдау ішекте түрлі бактериялардың әртүрлілігінің азайғанын көрсетті. Иммунитетке жауап беретін бактериялардың төмендеуі, 2 типті қант диабетінің, метаболизмдік синдромның және ішектің қабыну ауруларының дамуына бейім бактериялардың жоғары үлесі байқалады.

Түйін сөздер: ішек микробиомы, 16s рРНК гені.

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ИССЛЕДОВАНИЕ ВИДОВОГО СОСТАВА МИКРОБИОМА КИШЕЧНИКА С ИСПОЛЬЗОВАНИЕМ ТАРГЕТНОГО СЕКВЕНИРОВАНИЯ ГЕНА 16S рРНК

Аннотация. Микробиом кишечника является экстракорпоральным органом и характеризуется сложной иерархической структурой, которая выполняет разнообразные функции, зависящие от ее количественного и видового состава. Нарушение ее баланса может приводить к возникновению различных патологических состояний (атеросклероз, заболевания сердечно-сосудистой системы, воспалительные заболевания кишечника, сахарный диабет 2 типа и др.).

С целью изучения видового соотношения бактерий в кишечнике, особенностей бактериальных видов и энтеротипов было проведено молекулярно-генетическое исследование гена 16S рРНК микроорганизмов с применением технологии следующего поколения (NGS).

Используя метод полупроводникового секвенирования были получены первые результаты анализа видового состава микробиома кишечника 33 пациентов. Идентифицировано 5 основных филов: Actinobacteria (4,9%), Bacteroidetes (37%), Firmicutes (42%), Proteobacteria (16%) и Vericomicrobia (0,02%). Обнаружено 298 основных таксономических единиц.

Анализ показал, что в популяции бактерий в малом количестве представлены бактерии, которые являются индикаторами более здорового метаболического статуса кишечника.

При проведении филогенетического анализа бактериальной популяции идентифицировано 2 энтеротипа: у 58% пациентов -1 энтеротип Bacteroides, у 42% - 2 энтеротип Prevotella.

Метагеномный анализ видового соотношения бактерий показал, что в кишечнике наблюдается снижение видового разнообразия бактерий. Отмечается снижение бактерий, отвечающих за иммунитет, высокая доля бактерий, предрасполагающая к развитию сахарного диабета 2 типа, метаболического синдрома и воспалительных заболеваний кишечника.

Ключевые слова: микробиом кишечника, ген 16S рРНК.

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