ALTERED NEUROMETABOLIC POTENTIAL OF GUT MICROBIOME IN HEALTHY CHILDREN OF DIFFERENT AGE

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Recently much attention is paid to investigation of the gut microbiome impact on children's mental health. The study was aimed to detect alterations in the taxonomic composition and content of bacterial genes encoding key enzymes involved in the metabolism of neuroactive compounds in the metagenomes of healthy young children and adolescents. The whole metagenome sequencing was used to obtain the metagenomic data of the faecal specimens. The bioinformatics algorithm developed and the catalogue of homologs created were used to identify the changes in abundance of bacterial genes and metagenomic signatures in the studied metagenomes. The core neurometabolic signature of the healthy children gut microbiota included the *Bacteroides uniformis*, *Faecalibacterium prausnitzii* and *Lachnospiraceae bacterium* species, as well as genes involved in production of acetic, propionic and butyric acids, glutamate and enzymes possessing antioxidant activity. Comparison of metagenomes in children of different age groups revealed significant ($p < 0.1$) changes in the average abundance for 3 bacterial genera and 18 species. The higher alpha diversity of the adolescents' microbiota was observed both at the genus and species level. Furthermore, in the adolescents' microbiota metagenomes the increased average relative abundances for the genes encoding enzymes involved in production of SCFAs, glutamate, tryptophan and compounds with antioxidant properties, histidine degradation and linoleic acid conjugation were observed ($p < 0.1$). The study results support the evidence that healthy gut microbial communities become more diverse and functional as their human hosts become older.

Keywords: gut microbiota, gut-brain axis, metagenomic signatures, neurodevelopment, neuroactive compounds

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СТРУКТУРА И НЕЙРОМЕТАБОЛИЧЕСКИЙ ПОТЕНЦИАЛ МИКРОБИОТЫ КИШЕЧНИКА У ЗДОРОВЫХ ДЕТЕЙ РАЗНОГО ВОЗРАСТА

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В последние годы большое внимание уделяется изучению влияния кишечной микробиоты на здоровье детей, в том числе психическое. Целью данной работы было определить изменения в таксономическом составе и содержании бактериальных генов, кодирующих ферменты, участвующие в метаболизме нейроактивных соединений, в метагеноме микробиоты кишечника детей младшего и подросткового возраста. Данные для анализа были получены при помощи секвенирования полного метагенома. Для определения изменения представленности бактериальных генов и метагеномных сигнатур использовали разработанный биоинформатический алгоритм и каталог гомологов генов. В результате построена коровая нейрометаболическая сигнатура кишечной микробиоты здоровых детей младшего возраста, включающая в себя виды *Bacteroides uniformis*, *Faecalibacterium prausnitzii* и *Lachnospiraceae bacterium* и гены, участвующие в образовании уксусной, пропионовой и масляной кислот, глутамата и ферментов с антиоксидантной активностью. Сравнение метагеномов детей разных возрастных групп показало статистически значимое (P-value < 0,1) изменение представленности для 3 родов бактерий и 18 видов. Альфа-разнообразие микробиоты подростков выше как на родовом, так и на видовом уровнях. Кроме того, в микробиоте подростков повышена (P-value < 0,1) представленность генов, кодирующих ферменты, участвующие в образовании короткоцепочечных жирных кислот, глутамата, триптофана и ферментов с антиоксидантной активностью и деградации гистидина, конъюгации линолевой кислоты. Полученные результаты подтверждают имеющиеся данные об увеличении биоразнообразия и развитии функциональных свойств кишечного микробного сообщества со взрослением человека.

Ключевые слова: микробиота кишечника, ось кишечник-мозг, метагеномные сигнатуры, развитие нервной системы, нейроактивные соединения

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Today, human gut microbiota is considered an important organ, which plays a vital part in preserving human health. Gut microbiota is the microbial population colonizing the gastrointestinal tract. The healthy gut microbial communities, which contribute to preserving the metabolic homeostasis, live inside the host maintaining the immunological tolerance. Due to symbiotic relationship, human gut microbiota carries out various tasks contributing to the host's physiology [1]. The host co-evolves with the microbiota; the composition of gastrointestinal tract microbial community changes in response to various internal and external stimuli. Bacterial species colonizing the gastrointestinal tract in infancy affect the host's health later in life [2]. The gut microbiota's bacterial composition becomes stable after the first three years of life getting closer to the adult gut microbiota profile [3].

Clinical and experimental data demonstrate the significant impact of human gut microbiota on the broad range of behaviors, including the social behavior, mood, emotions, anxiety and nuitrition [4]. Gut bacteria affect various human neurological conditions via the microbiota-gut-brain axis [4]. The gut microbiota composition may affect the neural network formation during the early nervous system development [5]. Bacteria influence the central nervous system (CNS) and enteric nervous system (ENS) in a variety of ways via metabolites and hormones of immune system and afferent nerves. Bacteria produce hundreds of compounds, which may affect the host's physiology. The gut microbiota composition alterations may result in major changes in metabolite production. Since the host is constantly exposed to such molecules, those may facilitate the development of various neuropsychiatric disorders, including depression [6].

Adolescence and puberty are critical periods for the developing nervous system with numerous structural, neurochemical and molecular changes occurring in response to genetic and environmental signals. At this age microbiota is also subject to significant shifts in composition and functioning. Steroid hormones cause the sex-specific differences in the gut microbial composition. Maturation of human gut microbiota runs alongside with the dynamic brain development; both processes have similar critical periods of development [7].

The use of next-generation sequencing (NGS) provides a better understanding of gut microbiota composition and allows one to explore its structural changes throughout the lifespan [8]. During our work, we used the shotgun metagenomic sequencing to study the human gut microbiota. The method is based on sequencing of the complete genomic material of the microbiota sample, which makes it possible both to obtain full data on bacterial composition, and to assess the overall microbiota metabolic functions and the functional capabilities of all bacteria. Moreover, the method may be used for strain-level microbiota analysis. The study was aimed to detect alterations in the taxonomic composition and content of bacterial genes encoding key enzymes involved in the metabolism of neuroactive compounds and biomarker metabolites in the metagenomes of healthy children of different age groups: 3–5 years old (children's metagenomes (ChM) and 15 years old (adolescent metagenomes (AM).

METHODS

Cohorts and metagenome sequencing

The study included the previously sequenced gut microbiota metagenomes isolated from 23 healthy neurotypical children aged 3–5 (ChM group) [9] and 7 adolescents aged 15 (AM group) [10] living in Moscow Region. Inclusion criteria: age; no gastrointestinal disorder prior to sampling; geographic region of origin — Moscow and Moscow Region; no exposure to antibiotics, probiotics and prebiotics within 2 months before sampling; no mental disorder (depression, schizophrenia, bipolar disorder, etc.); no diarrhea. Faecal specimens obtained from each volunteer were stored in sterile plastic containers before analysis at a temperature of –80 °C.

Metagenomic DNA isolation, library construction and sequencing using the Illumina HiSeq system (Illumina; USA) were carried out in accordance with the previously reported algorithm [9]. Metagenomic reads were deposited in the Sequence Read Archive (SRA) NCBI (the ChM BioProject ID was PRJNA516054, and the AM ID was PRJNA380118). The raw sequence data quality control was performed using the FastQC tool, and the Trimmomatic tool was used for trimming [11, 12]. The bases with quality score Q < 20 and sequences shorter than 50 bp were removed. In order to remove human DNA, all reads were mapped to the human genome (hg19 assembly) using the bowtie2 tool [13]. The metagenomic reads were assembled into contigs using the metaSPADes software [14].

Parameters of the sequenced samples and the resulting assemblies are presented in Table 1.

Catalogue creation

The catalogue of gene homologs involved in synthesis and metabolism of various neuroactive compounds, which was introduced before [9, 15], was updated and expanded. The genes involved in synthesis and metabolism of various compounds and metabolites reported as biomarkers of depression were added to the catalogue [16]. The amino acid sequences of these genes' homologs were selected in accordance with the previously reported algorithm [9] (Table 2).

Taxonomic and statistical analysis

The taxonomic composition was defined using the Kraken2 [17] and TAGMA [18] software. The analyses were carried out separately for the taxonomic levels of phylum, genus and species. The alpha diversity (Shannon's diversity index) was assessed using the R programming language.

The significant differences in the taxonomic composition at the genus and species level were defined using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test (1000 permutations), the significance threshold was set to P-value < 0.1. This value was chosen due to the relatively small number of samples in the AM group.

Signature identification in metagenomic data

Metagenomic signature is a combination of genes found in the metagenome and bacteria containing such genes [9]. In order to define the signatures, the metagenomic assemblies were analyzed using the previously reported algorithm [15]. The search for open reading frames (ORF) was performed using the MetaGeneMark software (USA) [19]. The ORFs were annotated using the catalogue created and BLASTp with the following parameters: homology ≥ 60%; relative alignment score ≥ 80%. Bacterial origin of ORFs was defined at the taxonomic level of species using the Kraken2 software. All unclassified sequences were designated as "Unclassified". Thus, a set of pairs was obtained for each species (species; gene). In order to assess the relative abundance of the pair, the reads were mapped to the appropriate ORFs using the Burrows-Wheeler Alignment

Table 1. Characteristics of studied metagenomic samples

№	Reads			Assemblies		
	Group	Sample name	Size, billion base pairs	Size, Mb	Number of contigs	N50, bp
1	ChM	HC_1	2.99	160.03	197683	2827
2	ChM	HC_2	1.91	129.61	194544	3020
3	ChM	HC_3	2.63	166.00	210193	3795
4	ChM	HC_4	3.46	189.25	209685	7667
5	ChM	HC_5	1.85	154.83	238146	2148
6	ChM	HC_6	4.71	182.04	174019	4487
7	ChM	HC_7	5.16	178.69	194821	2532
8	ChM	HC_8	7.15	154.62	142565	3426
9	ChM	HC_9	5.86	256.49	259614	2096
10	ChM	HC_10	6.77	149.53	156608	2114
11	ChM	HC_11	5.06	192.06	153535	14058
12	ChM	HC_12	6.26	201.55	175868	9029
13	ChM	HC_13	6.09	168.69	141685	9188
14	ChM	HC_14	6.16	168.31	121753	6826
15	ChM	HC_15	7.2	226.17	236984	2053
16	ChM	HC_16	6.18	178.16	153050	4611
17	ChM	HC_17	5.83	280.50	311115	1871
18	ChM	HC_18	5.23	214.81	205380	3613
19	ChM	HC_19	5.26	140.45	116040	13178
20	ChM	HC_20	4.73	172.67	125060	15088
21	ChM	HC_21	8.31	248.61	205319	6630
22	ChM	HC_22	9.86	277.61	238778	7011
23	ChM	HC_23	8.51	172.41	163607	3065
24	AM	D3F	10.1	108.11	61735	9284
25	AM	D4F	9	241.5	152814	5677
26	AM	D5F	9.9	142.83	53487	21016
27	AM	D6F	8.6	206.81	202650	3438
28	AM	D11F	7.7	38.29	31806	13412
29	AM	D12F	7.5	76.07	130779	760
30	AM	D13F	10.6	180.43	335100	617

(BWA) tool [20]. The counts were normalized by the Trimmed Mean of M-values (TMM) method using the edgeR library [21]. The comparison of relative abundance values for the ChM and AM groups was carried out using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test (1000 permutations; P-value < 0.1).

RESULTS

Expanded gene catalogue

The previously constructed reference catalogue of gene homologs involved in the various neuromodulators metabolism has been expanded by adding genes, which encode enzymes involved in the new compounds production and the various neuroactive metabolites destruction [9, 15]. The resulting catalogue comprises 742 amino acid sequences for gene homologs encoding the 68 bacterial enzymes. The new enzymes involved in γ-aminobutyric acid (GABA), nitric oxide, γ-hydroxybutyric acid and p-cresol decomposition, isovaleric acid, inositol and glutamate synthesis and decomposition, as well as antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) have been added to the catalogue. The full list of genes included in the updated catalogue is presented in Table 2.

Core neurometabolic signature of the healthy children gut microbiota

In the first phase, the search for bacterial genes encoding the key enzymes involved in the synthesis of neuroactive compounds and biomarkers of depression, which may affect the development and functioning of the child's nervous system during his/her early life was carried out in the metagenomes of the ChM group (Fig. 1). Only those genes found in more than 50% of samples were taken into account. The most abundant genes were those encoding methylmalonyl-CoA decarboxylase (propionic acid production), phosphotransacetylase (acetic acid production), glutamate decarboxylase (GABA synthesis), gamma-aminobutyrate antiporter (GABA transport) and histidine ammonia-lyase (histidine destruction). Gene homologs involved in metabolic pathways of GABA, serotonin, melatonin, butyric acid, conjugated linoleic acid, spermidine, isovaleric acid, inositol, γ-hydroxybutyric acid, glutamate, creatinine, indole, tryptophan, superoxide dismutase, catalase and glutathione peroxidase were also detected.

Table 2. Updated catalogue of homologs

№	Enzyme	Function	Number of homologs
1	DOPA decarboxylase	Synthesis of serotonin, dopamine and norepinephrine	10
2	Glutamate decarboxylase	GABA synthesis	28
3	Gamma-aminobutyrate antiporter	GABA transport	20
4	4-Aminobutyrate aminotransferase (gabT, puuE), glycine amidinotransferase	GABA decomposition	17
5	Histidine decarboxylase	Histamine synthesis	13
6	Serotonin N-acetyltransferase	Decomposition of serotonin for synthesis of melatonin	24
7	Acetylserotonin O-methyltransferase	Synthesis of melatonin	8
8	Nitric oxide synthase	Nitric oxide formation	6
9	Nitric oxide dioxygenase, nitric oxide reductase (norB, norC)	Nitric oxide decomposition	13
10	Aromatic amino acid hydroxylases	Catecholamine synthesis	7
11	Monoamine oxidase	Decomposition of serotonin, dopamine and norepinephrine	5
12	Phosphotransacetylase	Acetic acid formation	43
13	Butyrate kinase	Butyrate synthesis	16
14	Butyryl-CoA dehydrogenase	Butyric acid synthesis	32
15	Lactoyl-CoA dehydratase, propionaldehyde dehydrogenase, methylmalonyl-CoA decarboxylase	Propionic acid formation	55
16	Linoleic acid isomerase	Linoleic acid conjugation	23
17	Spermidine synthase	Spermidine synthesis	26
18	Tyrosine decarboxylase	Synthesis of tyramine and dopamine	11
19	2-Oxoisovalerate dehydrogenase (alpha, beta), dihydrolipoyl dehydrogenase	Isovaleric acid synthesis (KADH pathway)	24
20	Aldehyde dehydrogenase, pyruvate decarboxylase	Isovaleric acid synthesis (KADC pathway)	11
21	Myo-inositol-1 (or -4) -monophosphatase, myo-inositol-1-phosphate synthase	Inositol synthesis	11
22	Myo-inositol 2-dehydrogenase	Inositol decomposition	13
23	4-Hydroxybutyrate dehydrogenase	Decomposition of γ-hydroxybutyric acid	13
24	Glutamate synthase (gltB, gltD)	Glutamate II synthesis	22
25	Glutamate mutase (glmS, glmE), methylaspartate ammonia-lyase	Glutamate II decomposition	24
26	4-Hydroxyphenylacetate decarboxylase	P-cresol synthesis	8
27	"4-Cresol dehydrogenase, Protocatechuate 3,4-dioxygenase (pcaG, pcaH)"	P-cresol decomposition	15
28	Creatinin amidohydrolase	Creatinin synthesis	5
29	D-lactate dehydrogenase	D-lactic acid formation	13
30	Glutathione synthetase (gshAB, gshB)	Glutathione synthesis	12
31	Glutathione S-transferase, glutathione reductase, gamma-glutamyl transpeptidase	Glutathione decomposition	35
32	Histidine ammonia-lyase	Histidine decomposition	20
33	Vinylphenol reductase	Synthesis of 4-ethylphenol	7
34	Tryptophanase	Metabolization of tryptophan into indole	7
35	Chorismate mutase	Prephenate synthesis	8
36	Prephenate dehydrogenase	Synthesis of 4-hydroxyphenylpyruvate	10
37	Tyrosine-specific transport protein	Tyrosine transport	6
38	Tyrosine aminotransferase	Tyrosine synthesis	6
39	Phenylalanine aminotransferase	Phenylalanine synthesis	3
40	Phenylalanine-specific permease	Phenylalanine transport	6
41	Tryptophan synthase (alpha and beta)	Tryptophan synthesis	26
42	Tryptophan-specific transport protein, tryptophan permease	Tryptophan transport	7
43	Superoxide dismutase ([Mn], [Fe], [Cu-Zn]), catalase, glutathione perxidase	Antioxidant	73

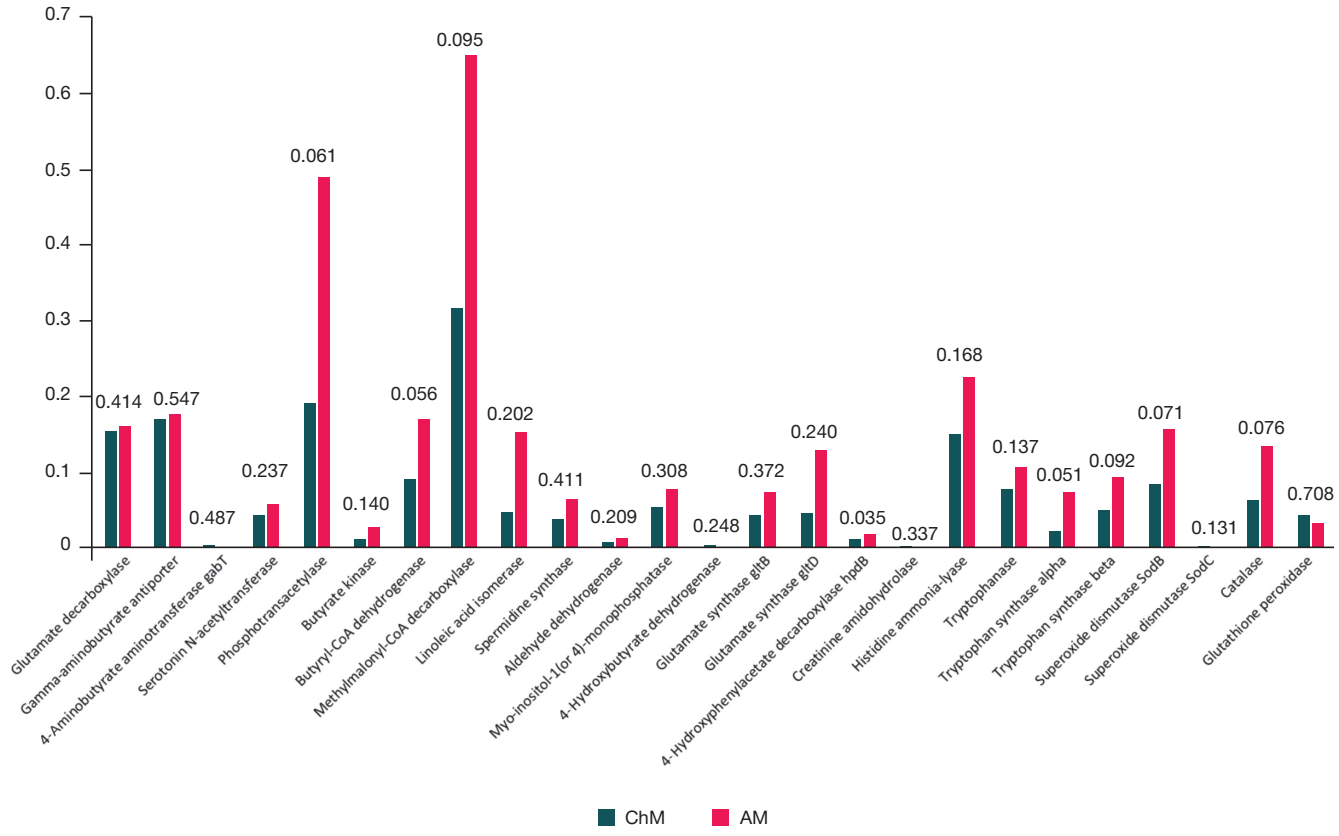

Fig. 1. Relative abundance of genes found in the ChM and AM groups. The figure shows the median relative abundance values for each gene found in more than 50% of samples. The values for the ChM group are *green* the values for the AM group are *red* The values in the head are P-values obtained using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test, 1000 permutations

Then, the bacterial origin of genes at the species level was defined, and the signature pairs were constructed (Fig. 2A). The pairs defined in the vast majority of samples (over 70%) comprised the core neurometabolic signature of the ChM (Fig. 2B). The ChM core neurometabolic signature included four species (*Bacteroides uniformis*, *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium и Parabacteroides distasonis*) and genes encoding 15 enzymes (glutamate decarboxylase, gamma-aminobutyrate antiporter, serotonin-N-acetyltransferase, phosphotransacetylase, butyrate kinase, butyryl-CoA dehydrogenase, methylmalonyl-CoA decarboxylase, linoleic acid isomerase, spermidine synthase, two subunits of glutamate synthase, histidine ammonia-lyase, tryptophanase, beta-subunit of tryptophan synthase and superoxide dismutase).

Age-related alterations in the neurometabolic signature of the human gut microbiota

Metagenomic samples for AM were analyzed using the same algorithm as the ChA samples. First, the search for gene homologs from the catalogue was carried out (see Fig. 1). On average, the increased relative abundance was noted for all genes found in more than 50% of the AM group samples compared to the ChM group. No homologs of genes encoding 4-aminobutyrate aminotransferase, creatinine amidohydrolase and superoxide dismutase (gene *sodC*) were found in the AM, however, this could be due to small sample size. Thus, the statistical power of the tests was low. The significant (adjusted P-value < 0.1) abundance increase was detected in AM for genes encoding phosphotransacetylase, butyryl-CoA dehydrogenase, methylmalonyl-CoA decarboxylase, 4-hydroxyphenylacetate decarboxylase, alpha- and beta-subunits of tryptophan synthase, superoxide dismutase (gene *sodB*) and catalase.

Then the metagenomic signatures for AM were constructed, and the abundance of pairs (species; gene) was compared with ChM (Fig. 2B). The significant abundance increase was detected for the following pairs: (*Alistipes onderdonkii*; catalase), (*A. onderdonkii*; glutamate decarboxylase), (A. onderdonkii; histidine ammonia-lyase), (*A. onderdonkii*; 4-hydroxyphenylacetate decarboxylase), (*Bacteroides vulgatus*; gamma-aminobutyrate antiporter), (*Bacteroides thetaiotaomicron*; methylmalonyl-CoA decarboxylase) and (*Barnesiella viscericola*; methylmalonyl-CoA decarboxylase). However, the observed alterations in the abundance of pairs comprising the core signature were not significant.

Comparative taxonomic analysis of gut microbiota in children of different age groups

All metagenomes were analyzed using the Kraken2 software. The alpha diversity comparison for ChM and AM is presented in Fig. 3. The average value of the Shannon's diversity index for AM was higher both at the genus (Fig. 3A) and species level (Fig. 3B).

The taxonomic composition of the ChM and AM was defined for the taxonomic levels of phylum, genus and species. At the phylum level, the AM were characterized by significant increase in abundance of Proteobacteria (8.99% vs. 3.37% in ChM and AM respectively, P-value = 0.001) (Fig. 3C). The differences were also revealed for phyla Actinobacteria (4.85% vs. 2.77%; P-value = 0.735), Bacteroidetes (60.55% vs. 66.94%; P-value = 0.421), Firmicutes (21.08% vs. 24.42%; P-value = 0.758) and Verrucomicrobia (0.40% vs. 1.36%; P-value = 0.298), however, the differences were not significant.

The comparison of abundance values at the genus (Table 3) and species (Table 4) level included only taxa identified in

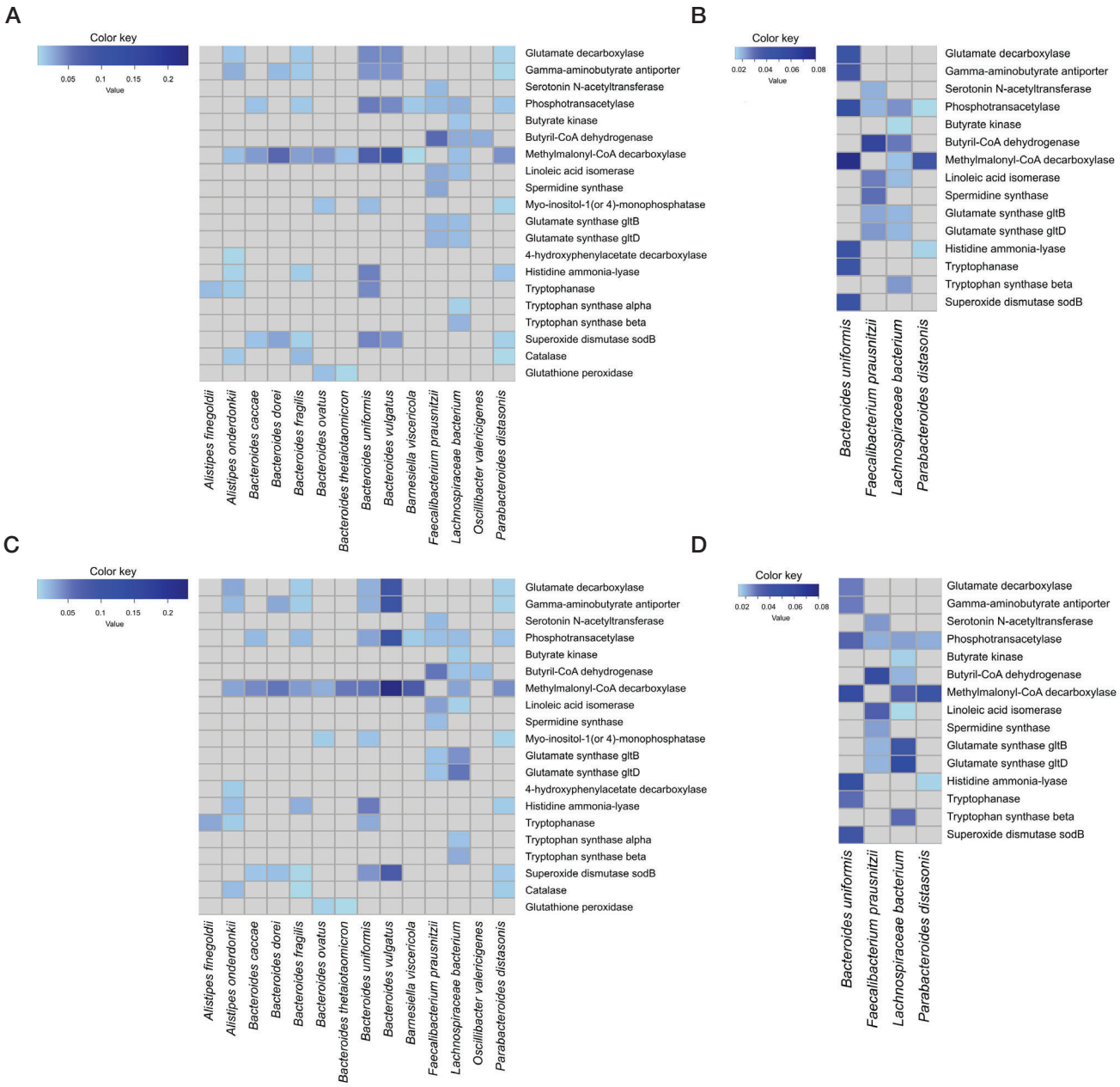

Fig. 2. Metagenomic signature and core metagenomic signature of human gut microbiota constructed for ChM group (**A** and **B** respectively), alterations of signature pairs abundance for AM group (**C** and **D** respectively). Color gradient shows the average relative abundance of the pairs (species; gene). Fig. (**A**) and (**B**) show only pairs found in more than 50% of samples, Fig. (**C**) and (**D**) show pairs found in more than 70% of samples

more than 50% of samples. As a result, the significant abundance increase (P-value < 0.1) was detected for genera *Butyrivibrio*, *Gordonibacter* and *Prevotella*. At the species level, there was a significant (P-value < 0.1) increase in abundance of *Alistipes communis*, *Alistipes megaguti*, *Alistipes sp*. dk3624, *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, *Eggerthella sp*. YY7918, *Lactobacillus reuteri*, *Lactobacillus ruminis*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella enoeca*, *Prevotella jejuni*, *Prevotella oris* and *Prevotella ruminicola*, and the decrease in abundance of *Bacteroides sp*. A1C1, *Gordonibacter pamelaeae*, *Enterococcus faecalis* and *Streptococcus thermophiles*.

In addition, the analysis of strain diversity for the studied samples was carried out using the TAGMA software (Russia) [18] (Table 5). In AM, the increased median number of strains compared to ChM was observed for the *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli* and *Streptococcus pneumonia* species. The larger number of strains per sample was also detected for the *Enterococcus faecium* species. However, the maximum number of strains was higher in the ChM group. Lower strain diversity in the AM group was observed for the *Bacteroides fragilis* species. Furthermore, in *Klebsiella pneumonia*, the median numbers of strains per sample were identical in both groups. However, the maximum number of strains was significantly higher in AM.

DISCUSSION

In order to study the possible mechanisms of the human gut microbiota impact on the early childhood neurostructural and neurocognitive development in healthy children, we focused on the group of bacterial genes encoding the enzymes involved in metabolism of neuroactive compounds, which correlate with dysregulations resulting in neurometabolic disorders and depression. The use of the constructed catalogue of homologs to genes of the selected group made it possible to

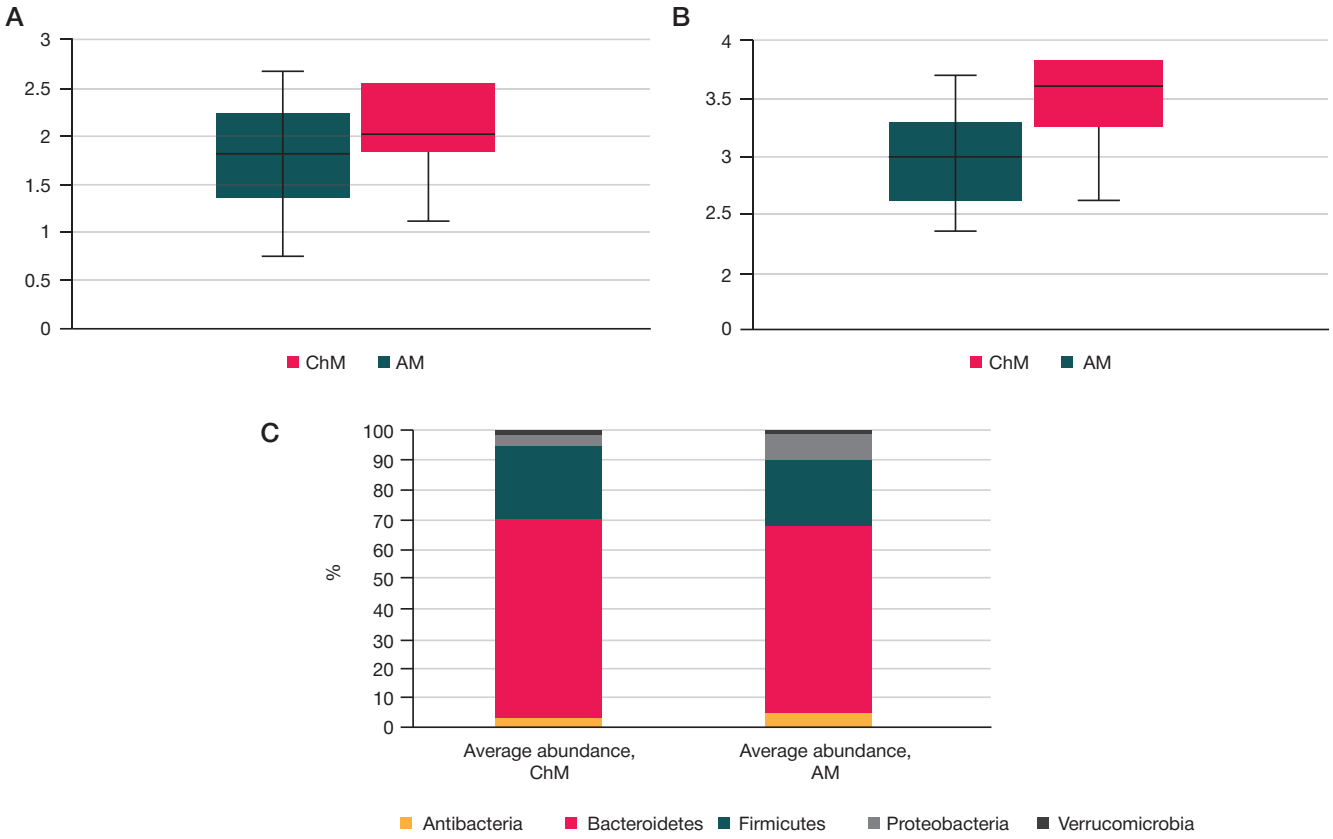

Fig. 3. Differences in taxonomic composition of gut microbiota for the ChM and AM groups. Alpha diversity for both groups was defined using the Shannon's diversity index at the genus (**A**) and species (**B**) level. The taxonomic composition alterations at the phylum level (**C**) are displayed as percentage values. The vertical error bars show the standard deviation

define the neurometabolic signature of the ChM. The signature approach was used to reveal the bacterial species comprising the largest number of genes (more than seven genes) responsible for production of various neuroactive compounds and thus having greater potential to affect the child's brain development and functioning. The revealed species were *B. uniformis*, *F. prausnitzii*, *L. bacterium* and *P. distasonis* being the gut commensals in healthy young children [22]. These bacteria contain genes encoding the proteins involved in production of acetic, propionic and butyric acids, GABA,

Table 3. Relative abundance of bacterial genera found in the ChM and AM groups

Species	Abundance in ChM group, %	Abundance in AM group, %	Ratio ChM/AM	Adjusted P-value	Proportion of identified samples (out of 30), %
Akkermansia	1.36 ± 3.33	0.38 ± 0.75	0.28	0.182	90
Alistipes	8.98 ± 7.60	7.22 ± 5.25	0.8	0.54	100
Bacteroides	49.16 ± 20.00	39.73 ± 18.84	0.81	0.261	100
Bifidobacterium	1.64 ± 2.93	3.08 ± 7.50	1.88	0.763	97
Blautia	1.05 ± 1.63	0.31 ± 0.16	0.3	0.237	100
Butyricimonas	0.33 ± 0.39	0.60 ± 0.84	1.84	0.232	100
Cupriavidus	0.02 ± 0.04	0.51 ± 1.30	22.3	0.178	80
Faecalibacterium	5.15 ± 4.85	3.42 ± 1.91	0.66	0.595	100
Flavonifractor	1.00 ± 1.57	0.24 ± 0.18	0.24	0.015	100
Lachnospira	1.22 ± 2.76	0.29 ± 0.29	0.24	0.18	100
Odoribacter	0.75 ± 0.79	1.28 ± 0.97	1.7	0.109	100
Parabacteroides	3.15 ± 4.03	2.63 ± 0.95	0.83	0.529	100
Paraprevotella	0.50 ± 0.89	0.88 ± 0.78	1.76	0.129	100
Phascolarctobacterium	1.05 ± 2.16	0.93 ± 1.26	0.89	0.652	57
Prevotella	0.22 ± 0.35	2.06 ± 3.31	9.24	0.033	100
Pseudomonas	0.17 ± 0.11	0.57 ± 0.40	3.43	1	100
Roseburia	1.40 ± 1.40	1.21 ± 1.23	0.87	0.485	100
Ruminococcus	1.59 ± 3.04	0.61 ± 0.82	0.39	0.457	97
Xanthomonas	0.07 ± 0.08	0.66 ± 0.59	10	1	100

Note: the table presents only genera found in more than 50% of samples with abundance of at least 0.5%.

Table 4. Relative abundance of bacterial species found in the ChM and AM groups

Species	Abundance in ChM group, %	Abundance in AM group, %	Ratio ChM/AM	Adjusted P-value	Proportion of identified samples (out of 30), %
Akkermansia muciniphila	1.35 ± 3.33	0.37 ± 0.75	0.28	0.127	90
Alistipes communis	0.94 ± 1.57	1.20 ± 0.91	1.27	0.075	100
Alistipes dispar	0.65 ± 1.39	0.29 ± 0.26	0.44	0.299	100
Alistipes finegoldii	2.55 ± 4.74	1.53 ± 2.25	0.6	0.662	100
Alistipes onderdonkii	2.65 ± 3.10	1.40 ± 2.10	0.53	0.3	100
Alistipes shahii	1.41 ± 2.37	1.82 ± 2.19	1.29	0.322	100
Bacteroides caccae	2.03 ± 3.24	2.21 ± 1.58	1.09	0.174	100
Bacteroides cellulosilyticus	1.69 ± 4.02	3.52 ± 6.67	2.09	0.111	100
Bacteroides dorei	7.92 ± 7.74	4.15 ± 3.17	0.52	0.358	100
Bacteroides fragilis	3.52 ± 3.84	1.62 ± 0.88	0.46	0.101	100
Bacteroides ovatus	4.87 ± 5.50	1.74 ± 0.97	0.36	0.218	100
Bacteroides sp. A1C1	1.77 ± 1.19	1.03 ± 0.67	0.58	0.05	97
Bacteroides sp. CBA7301	0.28 ± 0.34	0.75 ± 1.35	2.65	0.252	100
Bacteroides thetaiotaomicron	2.39 ± 2.29	1.38 ± 0.81	0.58	0.515	100
Bacteroides uniformis	5.87 ± 3.97	3.44 ± 2.51	0.59	0.109	100
Bacteroides vulgatus	7.78 ± 8.96	9.41 ± 10.14	1.21	0.54	100
Bacteroides xylanisolvens	2.17 ± 3.21	0.94 ± 0.83	0.43	0.629	100
Bifidobacterium adolescentis	0.41 ± 1.08	2.55 ± 6.60	6.17	0.227	93
Bifidobacterium longum	0.76 ± 2.14	0.19 ± 0.36	0.25	0.568	97
Blautia sp. SC05B48	0.87 ± 1.56	0.18 ± 0.10	0.21	0.315	100
Butyricimonas faecalis	0.33 ± 0.39	0.60 ± 0.84	1.84	0.208	100
Faecalibacterium prausnitzii	5.15 ± 4.85	3.42 ± 1.91	0.66	0.571	100
Flavonifractor plautii	1.00 ± 1.57	0.24 ± 0.18	0.24	0.012	100
Lachnospira eligens	1.22 ± 2.76	0.29 ± 0.29	0.24	0.151	100
Odoribacter splanchnicus	0.75 ± 0.79	1.28 ± 0.97	1.7	0.134	100
Parabacteroides distasonis	1.63 ± 2.03	1.35 ± 0.38	0.83	0.261	100
Paraprevotella xylaniphila	0.50 ± 0.89	0.88 ± 0.78	1.76	0.16	100
Roseburia intestinalis	0.99 ± 1.35	0.91 ± 1.08	0.92	0.878	100
Ruminococcus bicirculans	1.45 ± 2.99	0.41 ± 0.82	0.29	0.306	97
Xanthomonas euvesicatoria	0.05 ± 0.08	0.64 ± 0.58	11.73	1	100

Note: the table presents only species found in more than 50% of samples with abundance of at least 0.5%.

as well as antioxidant enzymes having a positive impact on the people's mental health. *B. uniformis*, *F. prausnitzii* and *L. bacterium* comprise the core neurometabolic signature of the healthy children gut microbiota, which may be used as an early childhood biomarker of normal microbiota.

Here we report the pilot study. This is the early stage of exploring the alterations in metabolic potential of gut microbiota in healthy children from early childhood to adolescence. So far, the small number of adolescents' samples was used for comparison. We had to determine whether the alterations in microbiota occurred during the child's development from infancy to adulthood. For this purpose, we compared taxonomic profiles and the content of bacterial genes encoding the key enzymes involved in the metabolism of neuroactive compounds.

We revealed differences in the quantitative content of bacterial genes responsible for production and destruction of neuroactive compounds in the compared metagenomes of children of different age groups, which was considered the most important result of the study. In AM, the two-fold increase in abundance of genes encoding the enzymes involved in the propionic, acetic and butyric acids, glutamate, and tryptophan production, histidine degradation, as well as in production of conjugated linoleic acid and antioxidant proteins was detected. As is known, all the listed above compounds have a positive impact on the gut and brain functioning, and contribute to homeostasis maintenance. The impact of the short-chain fatty acid levels on the energy homeostasis of the host has been reported [23]. Tryptophan is a substrate for the synthesis of neurotransmitter, serotonin [24]. Neurotransmitters serotonin and glutamate play a critical part in depression [25]. Conjugated linoleic acid and antioxidant proteins are the important factors of defense against oxidative stress. The content of other detected genes was negligible and demonstrated small differences. These data are likely to represent the varying contribution of the studied bacterial genes to maintaining the normal nervous system development in healthy children. The further transcriptome and metabolome analyses will be carried out to validate the results of the metagenomes bioinformatics analysis.

Earlier studies (based on the analysis of the 16S rRNA genes) aimed at the comparison of gut microbiota in children of different age groups revealed significant differences in the taxonomic composition [26]. In our study we used the data obtained by the shotgun metagenomic sequencing for the comparative metagenomic analysis. Although a small sample

Table 5. Strain diversity of bacterial species for the ChM and AM groups defined using the TAGMA software

Species	ChM		AM	
	Number of samples; proportion of samples (out of 23)	Average strains per sample [min; max]	Number of samples; proportion of samples (out of 7)	Average strains per sample [min; max]
Anaerostipes hadrus	20; 0.87	2 [1; 2]	7; 1.00	2 [1; 2]
Anaerotruncus colihominis	23; 1.00	1 [1; 2]	7; 1.00	1 [1; 1]
Bacteroides cellulosilyticus	22; 0.96	2 [1; 3]	7; 1.00	2 [1; 3]
Bacteroides clarus	22; 0.96	1 [1; 2]	6; 0.86	1 [1; 1]
Bacteroides dorei	14; 0.61	3 [3; 3]	5; 0.71	3 [1; 3]
Bacteroides faecis	18; 0.78	1 [1; 2]	4; 0.57	2 [1; 2]
Bacteroides finegoldii	21; 0.91	2 [1; 2]	7; 1.00	2 [2; 3]
Bacteroides fragilis	20; 0.87	7 [2; 11]	7; 1.00	2 [1; 11]
Bacteroides intestinalis	22; 0.96	2 [1; 2]	7; 1.00	2 [1; 2]
Bacteroides ovatus	22; 0.96	5 [1; 5]	7; 1.00	5 [1; 5]
Bacteroides vulgatus	21; 0.91	3 [1; 4]	7; 1.00	3 [2; 4]
Bacteroides xylanisolvens	21; 0.91	3 [1; 4]	7; 1.00	3 [2; 4]
Bifidobacterium adolescentis	14; 0.61	2 [1; 3]	6; 0.86	2 [1; 3]
Bifidobacterium longum	20; 0.87	4 [1; 6]	5; 0.71	4 [3; 5]
Blautia obeum	23; 1.00	3 [3–3]	7; 1.00	3 [3; 3]
Butyrivibrio crossotus	22; 0.96	2 [1; 2]	7; 1.00	2 [1; 2]
Catenibacterium mitsuokai	14; 0.61	1 [1; 1]	5; 0.71	1 [1; 1]
Clostridium asparagiforme	21; 0.91	1 [1; 1]	3; 0.43	1 [1; 1]
Clostridium botulinum	23; 1.00	2 [1; 6]	7; 1.00	3 [1; 5]
Clostridium pasteurianum	22; 0.96	1 [1; 2]	7; 1.00	1 [1; 2]
Clostridium perfringens	23; 1.00	2 [1; 5]	7; 1.00	4 [2; 5]
Clostridium sporogenes	17; 0.74	1 [1; 2]	5; 0.71	2 [1; 2]
Coprococcus catus	13; 0.57	1 [1; 1]	6; 0.86	1 [1; 1]
Coprococcus comes	19; 0.83	1 [1; 2]	6; 0.86	1 [1; 2]
Dialister invisus	14; 0.61	2 [1; 2]	4; 0.57	2 [1; 2]
Dorea formicigenerans	23; 1.00	2 [1; 3]	7; 1.00	2 [1; 3]
Eggerthella lenta	16; 0.70	2 [1; 2]	2; 0.29	2 [1; 2]
Enterococcus faecium	23; 1.00	1 [1; 8]	6; 0.86	2 [1; 2]
Escherichia coli	20; 0.87	10 [2; 54]	7; 1.00	25 [4; 40]
Eubacterium ramulus	22; 0.96	1 [1; 1]	7; 1.00	1 [1; 1]
Eubacterium rectale	23; 1.00	2 [1; 2]	7; 1.00	2 [1; 2]
Eubacterium ventriosum	13; 0.57	1 [1; 1]	5; 0.71	1 [1; 1]
Faecalibacterium prausnitzii	23; 1.00	5 [5; 5]	7; 1.00	5 [5; 5]
Klebsiella pneumoniae	17; 0.74	2 [1; 29]	6; 0.86	2 [1; 5]
Parabacteroides merdae	15; 0.65	3 [2; 3]	5; 0.71	2 [2; 3]
Roseburia intestinalis	23; 1.00	4 [4; 4]	7; 1.00	4 [4; 4]
Roseburia inulinivorans	23; 1.00	2 [1; 2]	7; 1.00	2 [2; 2]
Ruminococcus bromii	23; 1.00	1 [1; 2]	7; 1.00	1 [1; 2]
Ruminococcus gnavus	23; 1.00	2 [1; 2]	7; 1.00	2 [1; 2]
Ruminococcus lactaris	23; 1.00	1 [1; 1]	7; 1.00	1 [1; 1]
Ruminococcus torques	23; 1.00	2 [1; 2]	7; 1.00	1 [1; 2]
Streptococcus pneumoniae	14; 0.61	1 [1; 4]	3; 0.43	5 [1; 10]
Streptococcus suis	12; 0.52	1 [1; 1]	4; 0.57	1 [1; 1]
Veillonella parvula	12; 0.52	1 [1; 3]	3; 0.43	1 [1; 3]

of AM was used for comparison, the results obtained also demonstrated typical differences in the taxonomic composition of gut microbiota in children of different age groups. The significant increase in bacteria of the phylum *Proteobacteria* and no significant differences for phyla *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were revealed in AM. The alpha diversity of AM was higher both at the genus and species level, which was consistent with the published data on the more diverse microbiota of adolescents compared to young children [26]. High biodiversity often correlates with the higher content of probiotic bacteria. Our study revealed higher content of bifidobacteria (*B. adolescentis*) and lactobacilli in AM. It is

known that bifidobacteria and lactobacilli exhibit probiotic properties. Recently those were proposed as psychobiotics due to their ability to produce neuromodulators and affect the brain–gut interactions [27]. The significant relative content increase for the *Prevotella* genus representatives was detected in AM, as well as the decreased content of *A. muciniphila*, which demonstrated the negative correlation with obesity and inflammation [28]. Perhaps, the microbiota composition alterations observed in children as they mature are due to the impact of the diet and hormones. In turn, alterations in the composition if microbiota may affect the development of different brain areas [29].

Our findings revealed the strain diversity in both groups of metagenomes. The median increase of the bacterial strains in the AM for pathogenic bacteria *C. botulinum*, *C. perfringens*, *E. coli* and *S. pneumonia* was detected. Perhaps, that could be due to increased exposure to antibiotics during maturation. It is interesting that in the AM group there were more strains per sample (on average) for the *E. faecium* species, and less strains per sample for *B. fragilis*. Alterations in the strain-level microbiota composition may change its metabolic activity due to strain-specific production of various active compounds by bacteria. Combining the shotgun sequencing with the metagenome signature approach and the bioinformatics tools allowing one to perform the strain-level taxonomic analysis could put us closer to constructing the strain-level metagenomic signatures. This, in turn, would help to reveal the potential for specific production of neuroactive compounds in the new strains. This information could then be used to develop the methods for diagnosis of such neuropsychiatric disorders as depression, as well as to develop the targeted therapy for these disorders based on the use of pharmaceutical compounds, probiotics, prebiotics and/or psychobiotics [30].

CONCLUSION

The study results confirm and expand the knowledge that the gut microbial communities become more diverse and functional as their human hosts become older. The gut microbial communities significantly enrich themselves with genes involved in metabolism of neuroactive compounds and compounds possessing anti-inflammatory and antioxidant activity necessary for the nervous system function. These alterations occur in response to external and internal factors, such as diet, antibiotics, hormones, stress, etc. The detected neurometabolic signature of the healthy children gut microbiota may be used as a marker of the normal human gut microbiota condition. Future research should be focused on identification of the gut microbiota metagenomic signature in healthy children of different age groups from different backgrounds.

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