APPLICATION OF CULTURE-BASED, MASS SPECTROMETRY AND MOLECULAR METHODS TO THE STUDY OF GUT MICROBIOTA IN CHILDREN

Efimov BA ✉, Chaplin AV, Sokolova SR, Chernaia ZA, Pikina AP, Savilova AM, Kafarskaya LI

Pirogov Russian National Research Medical University, Moscow, Russia

In recent decades, nucleic acid sequencing technologies used for metagenomic analysis have become the main methods for assessing the composition of microbiota. At the same time, the use of novel methods of cultivation and identification of microorganisms in microbiological research led to the renaissance of culture-based technologies, because facilitated the discovery and isolation of both new strains of well-known microorganisms as well as uncultivated and unexplored bacterial taxa. The aim of this study was to evaluate the potential of using the culture-based method for the assessment of the qualitative and quantitative composition of the intestinal microbiota in healthy children. Eleven growth media were inoculated with serial dilutions of stool samples in order to analyze the profile of dominant anaerobic bacteria, as well as aerobic bacteria and fungi in 20 healthy children aged 2–4 years. The identification of microorganisms was performed using MALDI TOF MS and 16S rRNA gene fragment sequencing were used. 1,819 isolated and identified strains belong to 7 phyla, 13 classes, 18 orders, 33 families, 77 genera and 149 species in the *Bacteria* domain. The *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* phyla were most abundant and frequent. The greatest species diversity (more than 85 species) was found in the *Firmicutes* phylum. Ten new previously uncharacterized bacterial strains were isolated.

Keywords: gastrointestinal tract microbiota, children, isolation and purification of bacteria, biodiversity, microbiological techniques/methods, DNA sequencing, mass spectrometry, Matrix-Assisted Laser Desorption-Ionization

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✉ **Correspondence should be addressed:** Boris A. Efimov
Ostrovityanova 1, Moscow, 117997; efimov_ba@mail.ru

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ОПЫТ ПРИМЕНЕНИЯ КУЛЬТУРАЛЬНОГО, МАСС-СПЕКТРОМЕТРИЧЕСКОГО И МОЛЕКУЛЯРНОГО МЕТОДОВ В ИССЛЕДОВАНИИ КИШЕЧНОЙ МИКРОБИОТЫ У ДЕТЕЙ

Б. А. Ефимов ✉, А. В. Чаплин, С. Р. Соколова, З. А. Черная, А. П. Пикина, А. М. Савилова, Л. И. Кафарская

Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия

В последние десятилетия основными методами оценки состава микробиоты стали технологии секвенирования нуклеиновых кислот, используемые для метагеномного анализа. В то же время внедрение в практику микробиологических исследований новых методов культивирования и идентификации микроорганизмов привело к ренессансу культуральных технологий, поскольку позволило решить задачи по поиску и выделению новых штаммов как уже известных микроорганизмов, так и ранее некультивируемых и неизученных бактериальных таксонов. Целью работы было оценить потенциал использования культурального метода для оценки качественного и количественного состава кишечной микробиоты здоровых детей. Анализ состава доминирующих групп анаэробных бактерий, а также аэробных бактерий и грибов у 20 здоровых детей в возрасте 2–4 лет проводили путем высева серийных разведений фекалий на 11 питательных сред. Для идентификации микроорганизмов использовали метод MALDI TOF MS и секвенирование фрагмента гена 16S рРНК. Идентификация 1819 выделенных штаммов микроорганизмов показала, что они принадлежали к 7 типам, 13 классам, 18 порядкам, 33 семействам, 77 родам и 149 видам домена бактерий. По количеству и частоте встречаемости доминировали бактерии типов *Bacteroidetes*, *Firmicutes*, *Actinobacteria* и *Proteobacteria*. Наибольшее видовое разнообразие (более 85 видов) обнаружено среди бактерий типа *Firmicutes*. Выделено 10 штаммов новых, пока не охарактеризованных бактериальных видов.

Ключевые слова: микробиота кишечника, дети, выделение бактерий, биоразнообразие, микробиологические методы, секвенирование ДНК, масс-спектрометрия, MALDI TOF MS

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✉ **Для корреспонденции:** Борис Алексеевич Ефимов
ул. Островитянова, д. 1, г. Москва, 117997; efimov_ba@mail.ru

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Most representatives of human and animal intestinal microbiome are classified as difficult-to-cultivate or nonculturable groups of microorganisms. Currently, massive parallel sequencing of DNA samples is used predominantly for the assessment of the composition of gut microbiota, for example, sequencing of the fragments of genes encoding 16S rRNA or sequencing of genomic DNA fragments [1, 2]. However, it is often difficult to interpret the obtained data, since the analyzed nucleotide sequences sometimes cannot be correlated with known bacteria or bacteriophages [3–5]. These approaches also have a disadvantage: only the relative ratio of the dominant groups of bacteria can be characterized efficiently, while the exact number of dominant or minor taxa remains beyond such studies [6, 7]. Real-time PCR with species-specific or group-specific primers and subsequent normalization of the results using recombinant plasmid DNA containing the cloned regions of amplified gene fragments are used for more accurate quantitative determination of bacteria [8]. However, this method makes it possible to determine the total number of copies of amplified DNA regions in the sample rather than the number of viable bacterial cells. In addition, due to the complexity of the method, especially in the studies aimed at quantification of the wide range of microorganisms, this approach is mainly used to analyze the composition of large taxonomic clusters of microorganisms (genera, families, groups) rather than certain known species. Thus, along with the development of technologies based on the sequencing of the genetic material of microorganisms, it is still important to improve cultural methods, since it allows us to solve the problem of searching for, isolating, determining the number and studying the biological properties of new strains in well-known bacteria, as well as in unexplored bacterial taxa [9].

The aim of the study was to evaluate the potential of using the culture-based method to assess the qualitative and quantitative composition of the intestinal microbiota of healthy children by stool samples inoculation of growth media widely used in laboratory practice for fastidious bacteria.

METHODS

The study of the parameters of colon microbial colonization was carried out in a group of 20 healthy children of both sexes living in Moscow. 17 of them regularly attended preschool institutions, and three children were in home schooling. Children were selected by the authors of the study. The age of the subjects ranged from 2 years 11 months to 4 years 10 months (average age 3 years 5 months), of which there were 12 boys and 8 girls. Inclusion criteria: children of both genders; children's age 2.5–4 years; parental consent presence. Exclusion criteria: children of other age; the presence of any chronic disease, such as diabetes mellitus, bronchial asthma, gastrointestinal diseases (celiac disease, functional constipation, short bowel syndrome, or inflammatory bowel disease); the presence of food allergies or parental belief in lactose intolerance in a child; pronounced selectivity in food consumption; use of antibiotics, immunomodulatory, steroid or probiotic drugs for 6 months before the study; infectious gastroenteritis in the last 6 months before the study, confirmed by laboratory tests; history of gastrointestinal surgery.

The material for the study was the feces of children which were collected by the parents with a sterile spatula and placed in a sterile container for transportation. The study was carried out under the condition that the amount of material placed in a container was not less than 15 g, and the time of its delivery to the laboratory did not exceed 2 hours from the moment of collection. In the laboratory immediately after receiving the feces were homogenized, their tenfold serial dilutions (from 10 to 10^9 times) were prepared in test tubes with sterile Schaedler Anaerobe Broth liquid medium (Oxoid, Basingstoke; UK), and aliquots in a volume of 0.1 ml of the corresponding dilutions were inoculated on Petri plates with growth media. The isolation of strictly anaerobic bacteria was performed on the Schaedler Anaerobe Agar (Oxoid, Basingstoke; UK) with the addition of 5% (v/v) defibrinated sheep blood, Anaerobe Basal Agar (Oxoid, Basingstoke; UK) with the addition of sheep blood, Columbia Agar (bioMérieux, Marcy l'Etoile; France) with the addition of sheep blood. Inoculation of growth media was carried out from the 10^7, 10^8, and 10^9 fold dilutions of the sample. Bifidobacteria and sulfate-reducing bacteria were also isolated on the Bifidobacterium Agar (Himedia Labs Inc.; India) and Perfringens Agar Base (Himedia Labs Inc.; India) respectively from the specimen 10^5, 10^7 and 10^8 fold dilutions. The Petri dishes were incubated in the anaerobic jars (Schutt Labortechnik GmbH; Germany) filled with a gas mixture (85% N_2, 10% H_2, 5% CO_2) in the presence of platinum catalysts at 37 °C for 72 hours. Lactic acid bacteria were cultured on Lactobacillus MRS Agar medium (Himedia Labs Inc.; India) from the specimen 10^3 and 10^5 fold dilutions, the plates were incubated in the anaerobic jars (GasPak; USA) with the 7% CO_2 atmosphere for 48 hours. Aerobic bacteria were isolated from the sample 10, 10^3, 10^5 and 10^7 fold dilutions on the following media: Endo Agar (Becton Dickinson and Company, USA), Salmonella–Shigella-Agar (bioMerieux Marcy l'Etoile; France), Gelatin Mannitol Salt Agar (Staphylococcus Agar # 110, Himedia Labs Inc.; India), m-Enterococcus Agar (Difco Laboratories, Franklin Lakes; USA), Columbia Agar (bioMérieux, Marcy l'Etoile; France) with the addition of 5% (v/v) sheep blood. The Sabouraud Chloramphenicol 2 Agar medium (bioMérieux, Marcy l'Etoile; France) was used to isolate the fungi.

After incubation the culture properties of bacteria were described, morphological types were counted separately for each colony type. In addition, bacteria from each type of colonies were stained by Gram method, subcultured on plates with the same medium and incubated under anaerobic or aerobic conditions to obtain the stock of bacteria for identification and preservation. Partially, the isolated microorganism strains were lyophilized after freezing in a 10% sucrose/1% gelatin (w/v) solution in the Freeze Dryer SB1 (Chemlab; UK). Test tubes with lyophilized strains of microorganisms were stored at a temperature of –80 °C.

The primary identification of bacteria and fungi was performed using the MALDI TOF mass spectrometry on the Vitek MS Plus unit (bioMérieux; France) with the Saramis Premium v. 4.10 software according to the manufacturer's recommendations [10, 11]. The strains of bacteria, the species identity of which could not be established using MALDI-TOF mass spectrometry, were identified by 16S rRNA gene sequencing [12, 13]. In addition, 16S rRNA gene sequencing was used for some strains to confirm the results of species identification by mass spectrometry. The polymerase chain reaction (PCR) amplified a portion of the 16S rRNA gene using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3') for 35 cycles with the following program: denaturation 20 s at 94 °C; primer annealing 20 s at 58 °C; elongation 90 s at 72 °C. The obtained PCR product was purified using the Cleanup Standard kit (Evrogen; Russia). The Sanger sequencing of the amplified DNA fragment from the UF1 primer was carried out at Evrogen (Evrogen; Russia). The determination of the cut-off boundaries

of the sequences by the quality of electrophoregrams was carried out visually using the Chromas Lite software (version 2.6.6, Technelysium Pty. Ltd.; Australia). The species of bacteria was determined on the basis of a search for the nucleotide sequences obtained in the GenBank database using the Megablast algorithm. The result of the comparison was considered to correspond to the level of the species in the case when its partially sequenced 16S rRNA gene sequence resembled ≥ 98.7% of the sequence of the closest known bacterial species in the GenBank database [14].

The number of bacteria was expressed in $\log_{10}$ colony forming units in 1 g of the test material ($\log_{10}$ CFU/g). The CFU/g of the test material was calculated using the following formula: CFU/g = the number of colonies of the corresponding type of microorganisms grown on the plate (or the average number of colonies of the corresponding type of microorganisms, in cases when bacteria of one type gave growth on different media or gave growth only on one of growth media, but were determined at more than one dilution) × 10 × dilution ratio. The total number of cultured microorganisms per sample was calculated by adding the quantitative values of individual species.

Statistical data processing was performed using the Mann–Whitney test and Fisher's exact test, multiple comparisons were corrected using the Bonferroni method. The tendency to form clusters was checked using the VAT algorithm [15] and the principal component analysis.

RESULTS

In total, 1,819 strains of microorganisms were isolated from 20 healthy children. The species identification of most strains was carried out using the MALDI TOF MS. To establish the taxonomic identity of 140 bacterial strains that could not be identified by mass spectrometry 16S rRNA gene sequencing was performed. Comparative analysis of the obtained nucleotide sequences with the GenBank database showed that 130 bacterial strains belonged to 88 known species, and 10 belonged to new, not yet studied bacterial taxa. The number of identified microorganism species per sample varied from 21 to 48 and averaged 34 ± 8. The total number of viable bacteria per 1 g of faeces varied from 10.0 to 11.1 $\log_{10}$ CFU/g and averaged 10.6 ± 0.4 $\log_{10}$ CFU/g.

In general, it was found that the isolated strains belonged to 7 phyla, 13 classes, 18 orders, 33 families, 77 genera, and 149 species of the Bacteria domain. Also 3 species of fungi from 2 families of the *Saccharomycetales* order were identified.

The reduction in the dimension by principal component analysis, as well as the use of the VAT algorithm, did not reveal a tendency to form clusters from the microbiocenoses of the examined children on the basis of the obtained data on the quantitative and qualitative composition, which does not allow classifying the microbiocenoses in this study into enterotypes or their analogs. There were no statistically significant differences in the microbial composition of the gut tract microbiota, depending on the age and gender of children, which may be due to the small size and homogeneity of the sample.

Generic assignment, frequency of occurrence and the quantitative level of microorganisms isolated from feces of 20 healthy children are presented in tables 1–5. It was revealed that dominant by the number and frequency of occurrence bacteria belonged to the *Firmicutes* (9.8 ± 0.4 $\log_{10}$ CFU/g), *Bacteroidetes* (10.3 ± 0.4 $\log_{10}$ CFU/g), *Actinobacteria* (10.0 ± 0.5 $\log_{10}$ CFU/g), and *Proteobacteria* (8.5 ± 1.1 $\log_{10}$ CFU/g) phyla. Representatives of each of these groups of bacteria were found in all children. In addition, in 25% of children (an average of 9.1 ± 0.4 $\log_{10}$ CFU/g) the bacteria of the *Akkermansia muciniphila* species were obtained in pure culture, which belong to the *Verrucomicrobia* phylum, in two children the *Fusobacterium mortiferum* (8.8 and 8.6 $\log_{10}$ CFU/g) were isolated which represent the *Fusobacteria* phylum. From one child the strain of the *Victivallis vadensis* bacteria belonging to the *Lentisphaerae* phylum was isolated, its concentration was 10^9 $\log_{10}$ CFU/g.

The phylum *Actinobacteria* consisted of the two classes of bacteria: *Actinobacteria* and *Coriobacteriia* (Table 1). Bacteria of *Actinobacteria* class belonged to 4 orders and 4 families, the representatives of the *Bifidobacteriaceae* and *Propionibacteriaceae* families prevailed. *Bifidobacteria* (occurred in 100% of children), were the dominant microorganisms in the gut microbiota of healthy children. A total of 6 species of bifidobacteria were isolated, among them *B. longum*, *B. bifidum*, *B. adolescentis*, and bifidobacteria of the *B. catenulatum/pseudocatenulatum* group.

The *Coriobacteria* class was represented mostly by the families *Coriobacteriaceae* and *Eggerthellaceae*, the dominant species were *Collinsella aerofaciens* and *Eggerthella lenta*.

The phylum *Bacteroidetes* consisted of 5 bacteria families belonging to order *Bacteroidales* (Table 2). The *Bacteroidaceae* family was represented by single genus *Bacteroides*, to which the 14 identified species belong. The bacteroids were isolated in 100% of cases in average number 10.1 ± 0.4 $\log_{10}$ CFU/g of feces. Among bacteroids the *B. dorei/vulgatus* and *B. ovatus/xylanisolvens* species dominated in healthy children, as well as *B. uniformis*, *B. fragilis* and *B. thetaiotaomicron*.

The *Rikenellaceae* family was also represented by only one genus *Alistipes* and 9 isolated species. *Alistipes* occurred in 90% of healthy children. The average number of bacteria was 9.5 ± 0.4 $\log_{10}$ CFU/g, the dominating species were *A. onderdonkii*, *A. putredinis* and *A. finegoldii*.

The *Porphyromonadaceae* family bacteria were isolated from 75% of children, they belonged to the *Parabacteroides*, *Barnesiella* and *Coprobacter* genera. The dominant species of these taxa were *P.distasonis*, *P. merdae* and *B. intestinihominis* observed in 45%, 35% and 40% of children respectively. Moreover, in almost all cases when bacteria of these taxonomic groups were detected, their concentration in the specimen was equal to or was close to 10^9 CFU/g.

Bacteria of the *Prevotellaceae* family turned out to be the rarest representatives of the order *Bacteroidales* at the used threshold for the detection of anaerobic bacteria, which was at least 10^8 microbial cells per 1 g of feces. In total from three children (15%) 5 strains of bacteria belonging to the *Prevotella copri*, *P. melaninogenica*, *P. rara* and *Paraprevotella clara* species were isolated.

The phylum *Firmicutes* demonstrated the greatest diversity of taxa, it was represented by 4 classes of bacteria, including 7 orders, 17 families, 45 genera, and 93 species of microorganisms, including new bacterial taxa found in this study (Table 3). Class *Clostridia* was represented only by the order *Clostridiales*, which included 47 species of bacteria belonging to 30 genera and 6 families. Representatives of the *Lachnospiraceae* family were found in 85% of children in average concentration of 9.0 ± 1.0 $\log_{10}$ CFU/g and were the most common taxon of this class. Among the bacteria species belonging to this family and found more often than others, was *Clostridium clostridioforme*, observed in 55% of children at an average concentration of 8.2 ± 1.0 $\log_{10}$ CFU/g. The other common genera of the family *Lachnospiraceae* were *Blautia* (in 65% of children, average concentration 8.9 ± 0.9 $\log_{10}$ CFU/g), as well as bacteria belonging to the

Table 1. Species identity of the *Actinobacteria* phylum cultured bacteria of the gastrointestinal tract microflora isolated from healthy children (*n* = 20)

Phylum *Actinobacteria*			
Class *Actinobacteria*			
Taxa	Observed number (%)[a]	Mean ± SD $\log_{10}$ CFU/g[b]	Growth media[e]
Order *Bifidobacteriales*, family *Bifidobacteriaceae*, genus *Bifidoibacterium*	**20 (100)**	**9.8 ± 0.6**	
Bifidobacterium longum	20 (100)	9.3 ± 0.5	SAA; ABA; CA; BA
Bifidobacterium adolescentis	8 (40)	9.5 ± 0.6	SAA; ABA; CA; BA
Bifidobacterium catenulatum/pseudocatenulatum[c]	11 (55)	9.1 ± 0.7	SAA; ABA; CA; BA
Bifidobacterium bifidum	8 (40)	9.5 ± 0.6	SAA; ABA; CA; BA
Bifidobacterium animalis	6 (30)	9.3 ± 0.7	SAA; ABA; CA; BA
Bifidobacterium breve	2 (10)	9 – 10.2[d]	SAA; ABA; BA
Order *Propionibacteriales*, family *Propionibacteriaceae*, genus *Cutibacterium*	**7 (35)**	**9.1 ± 0.6**	
Cutibacterium acnes	5 (25)	8.9 ± 0.7	SAA; ABA; BA
Cutibacterium granulosum	2 (10)	9 – 9.8	ABA; CA
Class *Coriobacteriia*			
Order *Coriobacteriales*, family *Coriobacteriaceae*			
Collinsella aerofaciens	11 (55)	9.2 ± 0.7	SAA; ABA; CA; BA
Genus *Eggerthellales*, family *Eggerthellaceae*			
Eggerthella lenta	17 (85)	8.8 ± 0.7	SAA; ABA; CA
Gordonibacter pamelae	3 (15)	8.5 ± 0.9	ABA; CA; PAB
Raoultibacter massiliensis	1 (5)	9	CA
Slackia isoflavoniconvertens	1 (5)	9.3	CA
Adlercreutzia equolifaciens	1 (5)	9	CA

Note (used in this table and tables 2–5): [a] Frequency of occurrence (absolute number of subjects/percentage of subjects); [b] Mean ± standard deviation $\log_{10}$ of the number of viable microorganisms in 1 g of feces (CFU/g — colony forming units in 1 g of feces); [c] */* — A group of phylogenetically related microorganisms, identity was established using the MALDI TOF MS method on the Vitek MS Plus unit with the Saramis Premium V. 4.10 software; [d] Smaller and larger value of the $\log_{10}$ index of the number of viable microorganisms, if they were found only in two examined children in the group; [e] Names of the growth media used for isolation and registration of the relevant microorganisms types. SAA — Schaedler Anaerobe Agar; ABA — Anaerobe Basal Agar; CA — Columbia Agar; BA — Bifidobacterium Agar; PAB — Perfringens Agar Base; MRS — Lactobacillus MRS Agar; EA — Endo Agar; SSA — Salmonella–Shigella-Agar; GMSA — Gelatin Mannitol Salt Agar (Staphylococcus Agar # 110); mEA — mEnterococcus Agar; CAa — Columbia Agar, plates with which were incubated aerobically; SC2A — Sabouraud Chloramphenicol 2 Agar.

genus *Anaerostipes* (in 40% of children, concentration 8.2 ± 1.0 $\log_{10}$ CFU/g). Another common family of bacteria in the *Clostridia* class were the *Ruminococcaceae* representatives (in 65% of children, average concentration 8.8 ± 0.5 $\log_{10}$ CFU/g) mostly represented by the species *Flavonifractor plautii*, *Ruthenibacterium lactatiformans* and *Anaerotruncus colihominis*. It is worth noting that the bacteria of such species of the *Ruminococcaceae* family as *Faecalibacterium prausnitzii* and *Gemmiger formicilis*, which, according to the results of sequencing of the libraries of 16S rRNA genes, constitute the dominant part of the normal human gastrointestinal tract microflora [16], were isolated only from one child. Such results indicate that it is necessary to use selective growth media and exclude the specimen contact with atmospheric oxygen, due to the extremely high sensitivity of these bacterial taxa to the latter. Another frequently detected taxon of the *Firmicutes* phylum were members of the *Erysipelotrichaceae* family (class *Erysipelotrichia*, order *Erysipelotrichales*), which were found in 55% of healthy children at an average concentration of 8.5 ± 1.0 $\log_{10}$ CFU/g. Among 6 genera and 9 species of bacteria of this family, *Clostridium innocuum* and *Clostridium ramosum* dominated. They were detected with frequency equal to 40% in concentration exceeding 10^8 CFU/g.

Bacteria belonging to the *Negativicutes* class were present in 100% of children, the average concentration was 8.9 ± 0.7 $\log_{10}$ CFU/g. The dominant taxa of this group were representatives of the *Veillonellaceae* family, which included the bacteria of the genera *Veillonella* and *Dialister* (in 35% and 45% of children respectively), as well as the family *Acidaminococcaceae* mainly represented by the *Phascolarctobacterium faecium* species, isolated in high concentrations from 40% of children.

Among colon bacteria belonging to the phylum *Proteobacteria*, representatives of the *Betaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* classes were identified (Table 4). The *Betaproteobacteria* class included different bacteria species of the *Sutterellaceae* family (isolated from 60% of children, the average concentration 8.8 ± 0.4 $\log_{10}$ CFU/g). The *Deltaproteobacteria* was mainly represented by the hydrogen sulfide forming bacteria of the *Bilophila wadsworthia* species (isolated from 55% of children, the average concentration 8.0 ± 0.8 $\log_{10}$ CFU/g). Finally, the *Gammaproteobacteria* class was represented by only one family *Enterobacteriaceae*. *Escherichia coli* were determined in 100% of children at the average concentration of 7.2 ± 0.4 $\log_{10}$ CFU/g. Other relatively frequently observed members of the family were bacterial species *Enterobacter cloacae* (30%), *Citrobacter freundii* (20%) and *Klebsiella pneumoniae* (20%), their concentrations usually didn't exceed 10^6 CFU/g.

Fungi were found in 45% of healthy children in the amount of 3.4 ± 1.4 $\log_{10}$ CFU/g, all of the isolated strains belonged to the order *Saccharomycetales* (Table 5). In the feces of 35% of the children, fungi of the genus *Candida* of the family *Debaryomycetaceae* were identified. They belong mainly to the *C. albicans* species. In addition, in two children fungi of the *Clavispora lusitaniae* species belonging to the *Metschnikowiaceae* family were present.

The taxonomic properties of 10 strains of bacteria which were isolated during this study and the species identity of

Table 2. Species identity of the *Firmicutes* phylum cultured bacteria of the gastrointestinal tract microflora isolated from healthy children (*n* = 20)

Phylum *Firmicutes*			
Taxa	Observed number (%)	Mean ± SD $\log_{10}$ CFU/g	Growth media
Class *Erysipelotrichia*, order *Erysipelotrichales*			
Family *Erysipelotrichaceae*	**11 (55)**	**8.5 ± 1.0**	
Genus *Erysipelatoclostridium*	11 (55)	8.4 ± 0.9	
Clostridium ramosum	8 (40)	8.2 ± 0.9	SAA; ABA; CA; PAB
Clostridium innocuum	8 (40)	8.1 ± 0.43	SAA; ABA; CA; PAB
Clostridium saccharogumia	1 (5)	8.7	ABA
Clostridium spiroforme	1 (5)	9	SAA
Holdemanella biformis	1 (5)	9.4	SAA
Dielma fastidiosa	1 (5)	8	SAA; ABA; CA
Coprobacillus cateniformis	1 (5)	9	ABA
Absiella dolichum	1 (5)	8	CA
Turicibacter sanguinis	1 (5)	8	SAA
Class *Clostridia*, order *Clostridiales*			
Family *Clostridiaceae*	**10 (50)**	**8.5 ± 1.1**	
Genus *Clostridium*	4 (20)	8.1 ± 1.4	
Clostridium perfringens	3 (15)	8.5 ± 0.7	SAA; CA; PAB
Clostridium paraputrificum	2 (10)	6.3 – 9.4	SAA; ABA; PAB
Clostridium ventriculi	1 (5)	10.4	ABA
Clostridium barattii	1(5)	6	PAB
Hungatella hathewayi	7 (35)	7.8 ± 0.8	SAA; PAB
Mordavella sp.	1 (5)	9	ABA
Lactonifactor sp. ASD3451	1 (5)	8	PAB
Family *Lachnospiraceae*	**17 (85)**	**9.0 ± 1.0**	
Genus *Lachnoclostridium*	**13 (65)**	**8.4 ± 1.0**	
Clostridium clostridioforme	11 (55)	8.2 ± 1.0	SAA; ABA; PAB
Clostridium scindens	3 (15)	8.0 ± 0.0	SAA; PAB
Clostridium symbiosum	2 (10)	6.0 – 8.0	SAA; PAB
Lachnoclostridium sp. ASD2032	1 (5)	9	SAA
Clostridium lavalense	1 (5)	6	PAB
Clostridium hylemonae	1 (5)	9	SAA
Lachnoclostridium sp. ASD3950	1 (5)	9.3	ABA
Anaerostipes sp.	8 (40)	8.2 ± 1.0	SAA; ABA; CA
Eisenbergiella tayi	2 (10)	8.0 – 9.0	ABA; CA
Genus *Blautia*	**13/65**	**8.9 ± 0.9**	
Blautia torques	7 (35)	8.8 ± 0.5	SAA; ABA; CA
Blautia coccoides	6 (30)	7.5 ± 0.8	ABA; PAB
Blautia gnavus	6 (30)	8.7 ± 0.6	SAA; ABA; CA
Blautia luti	5 (25)	8.5 ± 0.7	SAA; ABA
Blautia faecis	5 (25)	8.6 ± 0.6	SAA; CA
Blautia obeum	3 (15)	8.5 ± 0.5	SAA; ABA
Blautia wexlerae	2 (10)	8.0 – 9.0	ABA; PAB
Blautia sp. ASD2945	1 (5)	8	ABA
Blautia caecimuris	1(5)	9.6	ABA; CA
Family *Ruminococcaceae*	**13/65**	**8.8 ± 0.5**	
Flavonifractor plautii	7 (35)	8.7 ± 0.5	SAA; ABA; PAB
Ruthenibacterium lactatiformans	5 (25)	8.5 ± 0.5	ABA; CA
Anaerotruncus colihominis	4 (20)	8.3 ± 0.5	ABA; PAB
Flavonifractor sp. ASD20665	1 (5)	7.3	PAB
Monoglobus pectinilyticus	1 (5)	8.3	SAA
Ruminiclostridium leptum	1 (5)	8	ABA

Faecalibacterium prausnitzii	1 (5)	8.3	ABA
Gemmiger formicilis	1 (5)	8	ABA
Agathobaculum sp. ASD2948	1 (5)	8	ABA
Ruminococcaceae ASD2818	1 (5)	8	SAA
Genus *Dorea*	**4 (20)**	**8.6 ± 0.6**	
Dorea *longicatena*	3 (15)	**8.2 ± 0.3**	SAA; ABA; PAB
Dorea *formicirans*	1 (5)	8	PAB
Dorea sp.	1 (5)	9.3	ABA
Sellimonas intestinalis	7 (35)	8.8 ± 0.5	SAA; ABA;
Fusicatenibacter saccharivorans	3 (15)	**8.4 ± 0.5**	ABA; CA
Coprococcus comes	1 (5)	8.8	ABA
Family *Eubacteriaceae*	4 (20)	7.8 ± 0.39	ABA; PAB
Eubacterium limosum			
Family *Christensenellaceae*	1 (5)	9	CA
Christensenella minuta			
Family *Peptostreptococcaceae*			
Terrisporobacter sp.	1 (5)	8	PAB
Paeniclostridium sordellii	1 (5)	9	SAA
Genera with uncertain taxonomic position			
Intestinimonas sp.	1 (5)	9	ABA
Lawsonibacter asaccharolyticus	1 (5)	9	CA
Class *Bacilli*			
Order *Lactobacillales*			
Family *Lactobacillaceae*			
Genus *Lactobacillus*	13/65	6.3 ± 1.6	
Lactobacillus casei/paracasei	6 (30)	6.3 ± 1.9	MRS
Lactobacillus gasseri/acidophilus	5 (20)	7.0 ± 1.2	MRS
Lactobacillus rhamnosus	3 (15)	4.5 ± 0.2	MRS
Lactobacillus salivarius/delbruekii	2 (10)	5.5 – 6.3	MRS
Lactobacillus fermentum	1 (5)	4	MRS
Lactobacillus brevis	1 (5)	4	MRS
Family *Leuconostocaceae*	1 (5)	6.3	MRS
Leuconostoc lactis			
Family *Enterococcaceae*			
Genus *Enterococcus*	16/80	6.1 ± 1.3	
Enterococcus faecalis	8 (40)	5.8 ± 1.0	mEA
Enterococcus faecium	9 (45)	4.9 ± 0.7	mEA
Enterococcus durans	3 (15)	4.3; 4.8; 9.5	mEA
Enterococcus avium/raffinosus	7 (35)	6.5 ± 0.8	mEA
Enterococcus casseliflavus	1 (5)	6	mEA
Enterococcus gallinarum	1 (5)	4	mEA
Family *Streptococcaceae*			
Genus *Streptococcus*	19/95	7.5 ± 1.2	
Streptococcus salivarius	17/85	6.9 ± 1.2	mEA. MRS
Streptococcus parasanguinis	9 (45)	6.6 ± 1.0	mEA. MRS
Streptococcus oralis/pneumoniae/mitis	5 (45)	7.1 ± 2.6	mEA. MRS
Streptococcus anginosus	2 (10)	5.0 – 5.7	mEA. MRS
Streptococcus mutans	2 (10)	6.1 – 6.3	mEA. MRS
Streptococcus constellatus	1 (5)	6	MRS
Streptococcus infantarius	1 (5)	8.4	MRS
Streptococcus disgalactiae	1 (5)	8.9	CA
Genus *Lactococcus*	1 (5)	6.1	MRS
Lactococcus lactis			

Family *Aerococcaceae*	1 (5)	6	SAA
Aerococcus viridans			
Order *Bacillales*			
Family *Staphylococcaceae*	18/90	5.0 ± 1.7	
Staphylococcus aureus	14/70	3.9 ± 0.8	GMSA
Staphylococcus epidermidis	6 (30)	4.3 ± 1.4	GMSA
Staphylococcus hominis	3 (15)	4.6 ± 0.8	GMSA
Staphylococcus haemolyticus	3 (15)	2.5 – 5.2	GMSA
Staphylococcus sacharolyticus	2 (10)	9	ABA
Staphylococcus warneri	2 (10)	5.2 – 8.8	GMSA; CA
Staphylococcus capitis	1 (5)	4.3	GMSA
Staphylococcus gallinarum	1 (5)	3.2	GMSA
Family *Bacillaceae*	6 (30)	3.4 ± 0.3	GMSA
Bacillus sp.			
Class *Negativicutes*			
Order *Veillonellales*	**12 (60)**	**8.8 ± 0.9**	
Family *Veillonellaceae*			
Veilonella sp.	7 (35)	8.0 ± 0.9	SAA; ABA; CA
Allisonella histaminiformans	1 (5)	8	ABA
Genus *Dialister*	**9 (45)**	**9.0 ± 0.5**	
Dialister invisus	8 (40)	9.1 ± 0.4	SAA; ABA
Dialister succinatiphilus	1 (5)	8	CA
Order *Selenomonadales*, family *Selenomonadacea*			
Megamonas sp.	2 (10)	8.8 – 9.0	ABA; CA
Order *Acidaminococcales,* family *Acidaminococcaceae*			
Phascolarctobacterium faecium	8 (40)	9.0 ± 0.4	SAA; ABA; CA
Phascolarctobacterium succinatutens	1 (5)	8	SAA

which could not be established are listed in the Table 6. Most of them (7 of 10 strains) belonged to the phylum *Firmicutes*. Four of them had a phylogenetic relationship with species from the genera *Blautia*, *Flintibacter* and *Lachnoclostridium* of the family *Lachnospiraceae*. Two clones were close to different species of the family *Ruminococcaceae*, and another clone was phylogenetically similar to members of the genus *Lactonifractor* of the family *Clostridiaceae*. In addition, one clone of the new bacterial taxon was associated with a typical strain belonging to the family *Sutterellaceae*, included in the *Proteobacteria* phylum, and one clone belonged to the genera *Parabacteroides* and *Bacteroides* (families *Porphyromonadaceae* and *Bacteroidaceae* respectively, phylum *Bacteroidetes*).

DISCUSSION

This age group of children was chosen for the study because earlier it was shown that the qualitative and quantitative parameters of the gut microbiota by the age of three years become close to the typical adults values [17]. By this age, the gut microbiota acquires relative compositional stability and does not change significantly over time [18].

The main problems regarding the culture-based methods are associated with the selection of growth media, which provide the growth of fastidious strictly anaerobic bacteria, and with the further identification of numerous strains of microorganisms growing on these media. In our study we used well-known growth media including media for the strictly anaerobic bacteria. After inoculation of media Petri dishes were incubated at 37 °C in the anaerobic jars.

It was previously shown that increase the number of growth media and the number of samples leads to isolation of a large number of bacterial species, which indicates significant individual differences in the composition of the human gut microbiota [3]. This is confirmed by the results of metagenomic sequencing, which revealed a very high variability in abundance (12–2,200 times) for the 57 most common human bacterial species [19]. In our study, though an average of 34 ± 8 species of microorganisms was isolated from each child, in total, 159 species of bacteria were found in all children, including new taxa.

The greatest species diversity (more than 90 species of bacteria) was found in the phylum *Firmicutes*, with more than half of them belonging to the class *Clostridia* of the order *Clostridiales*. The dominating by frequency of occurrence and quantitative content families of this class were *Lachnospiraceae* and *Ruminococcaceae*. The obtained data characterizing the composition of this part of microbiota in Russian children correspond with the results of previous studies, in which both culture-based methods and the analysis of the nucleotide sequences of the libraries of 16S rDNA genes established the dominance of these taxa in human gut microbiota [6, 20].

Among the representatives of intestinal endosymbionts, which belong to the classes *Erysipelotrichia* and *Clostridia* isolated in pure culture from feces of healthy children the bacteria associated with various infectious diseases were also present. Therefore, the improvement of the culture methods and species identification for the bacteria of these taxa has not only ecological but also clinical significance. For example, *Clostridium innocuum* is often associated with bacteriemia in

Table 3. Species identity of the *Bacteroidetes* phylum cultured bacteria of the gastrointestinal tract microflora isolated from healthy children (n = 20)

Phylum *Bacteroidetes*, class *Bacteroidia*, order *Bacteroidales*			
Taxa	Observed number (%)	Mean ± SD $\log_{10}$ CFU/g	Growth media
Family *Bacteroidaceae*	**20 (100)**	**10.1 ± 0.4**	
Bacteroides dorei/vulgatus	19 (95)	9.5 ± 0.5	SAA; ABA; CA
Bacteroides ovatus/xylanisolvens	16 (80)	9.2 ± 0.5	SAA; ABA; CA
Bacteroides uniformis	17 (85)	9.4 ± 0.5	SAA; ABA; CA
Bacteroides fragilis	9 (45)	9.1 ± 0.6	SAA; ABA; CA
Bacteroides thetaiotaomicron	9 (45)	9.1 ± 0.5	SAA; ABA; CA
Bacteroides caccae	8 (40)	9.0 ± 0.7	SAA; ABA; CA
Bacteroides eggerthii	6 (30)	9.2 ± 0.5	SAA; ABA; CA
Bacteroides stercoris	4 (20)	9.0 ± 0.2	SAA; ABA; CA
Bacteroides intestinalis	3 (15)	9.2 ± 0.3	SAA; ABA; CA
Bacteroides clarus	2 (10)	9.0 – 10.3	ABA
Bacteroides massiliensis	2 (10)	9.3 – 9.5	SAA; ABA; CA
Bacteroides plebeius	1 (5)	9.6	SAA; ABA; CA
Bacteroides coprocola	1 (5)	8	ABA
Bacteroides salyersiae	1 (5)	8	CA
Bacteroides sp. ASD2038	1 (5)	9.7	SAA
Family *Rikenellaceae*	**18/90**	**9.5 ± 0.4**	
Alistipes onderdonkii	11 (55)	9.1 ± 0.5	SAA; ABA; CA
Alistipes putredinis	10 (50)	9.5 ± 0.5	SAA; ABA; CA
Alistipes finigoldii	8 (40)	8.8 ± 0.7	SAA; ABA; CA
Alistipes shachii	5 (25)	9.2 ± 0.3	SAA; ABA; CA
Alistipes indistinctus	2 (10)	8.0 – 9.0	CA
Alistipes obessii	3 (15)	9.1 ± 0.2	ABA
Alistipes inops	3 (15)	8.8 ± 0.7	SAA; ABA
Alistipes massiliensis	1 (5)	8.8	ABA; CA
Alistipes ihumii	1 (5)	9.4	SAA; ABA
Family *Porphyromonadaceae*	**15 (75)**	**9.4 ± 0.5**	
Genus *Parabacteroides*	13 (65)	9.2 ± 0.7	
Parabacteroides distasonis	9 (45)	9.1 ± 0.8	SAA; ABA; CA
Parabacteroides merdae	7 (35)	8.9 ± 0.7	SAA; ABA; CA
Parabacteroides sp. ASD2049	1 (5)	9	SAA
Barnesiella intestinihominis	8 (40)	9.2 ± 0.3	SAA; ABA; CA
Coprobacter fastidiosus	3 (15)	9.1 ± 0.1	SAA; ABA; CA
Family *Odoribacteraceae*	**8 (40)**	**9.3 ± 0.3**	
Odoribacter splanchnicus	5 (25)	9.3 ± 0.3	SAA; ABA; CA
Butyricimonas sp.	4 (20)	9.3 ± 0.4	ABA; CA
Family *Prevotellaceae*	**3 (15)**	**8.8 ± 0.9**	
Prevotella copri	2 (10)	8.5 – 9.0	SAA; ABA; CA
Prevotella melaninogenica	1 (5)	8	ABA
Prevotella rara	1 (5)	9	ABA; CA
Paraprevotella clara	1 (5)	9.6	ABA; CA

patients with immunodeficiency. It is resistant to antibacterial drugs used for treatment of anaerobic infections. *C. ramosum*, which were also isolated, are considered the second most common bacteria from the clostridium group after *C. perfringens*, causing abscesses, peritonitis, bacteriemia and chronic otitis media in children, and the third most common type of clostridia causing bacteriemia in adults [21, 22].

The *Bacteroidetes* phylum made up the second group by the number of identified taxa after *Firmicutes* and was represented by 33 species of bacteria belonging, however, to only one order *Bacteroidales*.

It is known that *Bacteroidales* includes the main part of anaerobic nonsporeforming gram-negative rod-shaped bacteria that colonize the human gastrointestinal tract [23]. We found, that in the group of children under study representatives of the *Bacteroidaceae*, *Rikenellaceae* and *Porphyromonadaceae* families dominated and were isolated from 100%, 90% and 75% of children respectively. In our previous study, to assess

Table 4. Species identity of the *Proteobacteria* phylum cultured bacteria of the gastrointestinal tract microflora isolated from healthy children (*n* = 20)

Phylum *Proteobacteria*			
Class *Gammaproteobacteria*			
Taxa	Observed number (%)	Mean ± SD log_{10} CFU/g	Growth media
Order *Enterobacteriales*, family *Enterobacteriaceae*			
Escherichia coli	20 (100)	7.2 ± 1.4	EA; CAa
Enterobacter cloacae	6 (30)	6.0 ± 1.5	EA; SSA; CAa
Citrobacter freundii	4 (20)	5.8 ± 0.68	EA; SSA; CAa
Klebsiella pneumonia	4 (20)	6.5 ± 1.7	EA; CAa
Leclercia adecarboxylata	1 (5)	6.5	CAa
Proteus mirabilis	1 (5)	6	SSA; CAa
Class *Betaproteobacteria*, order *Burkholderiales*			
Family *Sutterellaceae*	12 (60)	8.8 ± 0.4	
Parasutterella excrementihominis	4 (20)	8.6 ± 0.4	ABA; CA
Sutterella wadsworthensis	5 (25)	8.9 ± 0.1	ABA
Sutterella massiliensis	1 (5)	9.5	CA
Sutterella sp. ASD3426	1 (5)	8	CA
Duodenibacillus massiliensis	1 (5)	9.1	SAA
Family *Oxalobacteraceae*	1 (5)	8	CA
Massilia timonae			
Class *Deltaproteobacteria*			
Order *Desulfovibrionales*, family *Desulfovibrionaceae*			
Bilophila wadsworthia	11 (55)	8.0 ± 0.8	SAA; CA; PAB
Desulfovibrio piger	1 (5)	8.1	PAB

the composition of the dominant groups of intestinal bacteria belonging to the *Bacteroidales* order in 6 years old children, we inoculated serial dilutions of feces only on Columbia Blood Agar with the addition of sheep blood followed by determining the species of anaerobic gram-negative rod-shaped bacteria using the restriction analysis of amplified fragments of the 16S rRNA gene (ARDRA), as well as their sequencing.

In that study, we isolated only 38 strains of bacteria belonging to 13 species of *Bacteroidales* from 8 children [12]. In the present study, we used 3 different growth media to identify the same group of bacteria, with preliminary identification of all the grown bacteria using mass spectrometry and additional 16S rDNA gene sequencing for strains with an unclear taxonomic position. This approach allowed us to distinguish 33 species of bacteria belonging to 9 genera and 5 families of the order *Bacteroidales* in addition to bacteria belonging to other taxonomic groups.

Bacteria of *Prevotellaceae* family, also belonging to the order *Bacteroidales*, were found only in three children (15%). In spite of the fact that about 30 prevotella's species are known to date, as bacteria colonizing mainly the human oral cavity, until recently only 2 species *P. copri* and *P. stercorea* were considered commensals of the gastrointestinal tract. In our study in addition to *P. copri* the bacteria from species *P. melaninogenica*, which are rarely isolated from the intestine, and recently described new species *P. rara* were isolated from a child for the first time [24].

As predominant groups of intestinal bacteria, prevotella is most often determined in people whose diet is based on the products of plant origin, which is associated with the ability of these bacteria to degrade plant polysaccharides in the distal intestinal tract [20]. On the other hand, the prevalence in the gut microbiota the bacteria of the *Bacteroides* genus and the *Clostridiales* order, as shown in our study, had been previously associated with a mixed diet characterized by the inclusion in diet both animal and vegetable products along with easily digestible carbohydrates [25, 26].

CONCLUSIONS

The approach we used, based on the use of a wide range of growth media for the isolation of difficult-to-cultivate groups of intestinal endosymbionts, both under aerobic and anaerobic conditions, followed by the bacteria species identification by the MALDI TOF mass spectrometry and the 16S rRNA gene sequencing, allowed us to analyze the qualitative and quantitative composition of the dominant cultivated groups of gut microbiota in children. In general, the results describing the taxonomic composition of the fecal microbiota of children obtained by the culture-based method do not contradict the data obtained by molecular methods based on the sequencing of bacterial DNA. In addition, we have isolated in pure culture the numerous strains of difficult-to-cultivate bacteria and 10 strains of new bacteria with not yet studied biological properties. The data obtained allow us to expand our understanding of the spectrum of cultivated taxonomic groups of colon bacteria and their quantitative content in children. The strains we isolated, both belonging to known and new taxa, can be used to study a wide repertoire of their properties, including their biotherapeutic potential for creating the new probiotic medications. At the same time, many well-known taxa of gut microbiota, including representatives of such dominant genera as *Faecalibacterium* and *Roseburia*, we were unable to isolate, which indicates the need to use

Table 5. Species identity of the *Saccharomycetales* order fungi isolated from healthy children (*n* = 20)

Fungi taxa	Observed number (%)	Mean ± SD log_{10} CFU/g	Growth media
Order *Saccharomycetales*	**9 (45)**	**3.4 ± 1.4**	
Family *Debaryomycetaceae*	**7 (35)**	**3.5 ± 1.4**	
Candida albicans	5 (25)	3.7 ± 1.4	SC2A
Candida parapsilosis	1 (5)	2.5	SC2A
Candida sp.	1 (5)	2.5	SC2A
Family *Metschnikowiaceae*	2 (10)	2.0 – 5.0	SC2A
Clavispora lusitaniae			

Table 6. New gastrointestinal tract bacteria phylotypes isolated from healthy children and the values of the nucleotide sequences of their 16S rDNA levels of homology with the same sequences of typical strains of the most closely related validated species in accordance with the International Code of Nomenclature of Bacteria (Bacteriological Code)

№	Strain number	Phylum/family	№ of sequencing in the GenBank database	Related strains with high level of sequence similarity (megablast algorithm) in the GenBank database	Homology (%)
1	ASD 3426	*Proteobacteria Sutterellaceae*	MK615133.1	*Sutterella wadsworthensis* WAL9799	97.93
2	ASD2049	*Bacteroidetes Porphyromonadaceae*	MG321612.1	*Parabacteroides merdae* JCM9497	96.63
3	ASD2038	*Bacteroidetes Bacteroidaceae*	MK615124.1	*Bacteroides ovatus* ATCC 8483	98.19
4	ASD2032	*Firmicutes Lachnospiraceae*	MK615123.1	[Clostridium] *amygdalinum* BR-10	95.72
5	ASD2945	*Firmicutes Lachnospiraceae*	MK615128.1	*Blautia faecis* KB1	96.03
6	ASD3950	*Firmicutes Lachnospiraceae*	MK615131.1	[Clostridium] *glycyrrhizinilyticum* ZM35	95.73
7	ASD3451	*Firmicutes Clostridiaceae*	MK615130.1	*Lactonifactor longoviformis* ED-Mt61/PYG-s6	94.61
8	ASD20665	*Firmicutes Ruminococcaceae*	MK615126.1	*Flintibacter butyricus* BLS21	97.33
9	ASD2818	*Firmicutes Ruminococcaceae*	MH043116.1	*Caproiciproducens galactitolivorans* BS-1	93.76
10	ASD2948	*Firmicutes Ruminococcaceae*	MK615129.1	*Agathobaculum desmolans* ATCC43058	97.01

more advanced anaerobic technologies in such studies, in particular, anaerobic glove box at the stages of sample preparation, inoculation and grown culture counting. The complexity and labor input of the culture-based method do not allow recommend it for the routine use in clinical practice, even assuming that in the future all stages of the study would be fully automated. However, the development of methods of isolation and identification of strictly anaerobic colon bacteria is necessary, since these bacteria can have pathobiotic potential and occur in clinical material (wound discharge, biopsy specimens, blood, liquor, etc.), in which the usual quantitative content of the species of bacteria is not so great.

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