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ORIGINAL ARTICLE

Comparative gas chromatography-mass spectrometry study of the composition of microbial chemical markers in feces

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Abstract

Gas chromatography-mass spectrometry analysis was used to determine fatty acids, the markers of microorganisms, in the feces, including neonatal transitional stool and meconium, and healthy adults of different ages. It revealed the markers of *Eubacterium, Clostridium, Bifidobacterium, Lactobacillus, Enterococcus, Rhodococcus, Streptomyces, Enterobacteriaceae, Bacteroides, Helicobacter pylori, Alcaligenes, Peptostreptococcus, Candida, Streptococcus, Staphylococcus, Fusobacterium* sp. and other bacteria, as well as yeast, microscopic fungi, and viruses. The fecal microbial concentration was estimated to be within (0.3–4) $\times$ 10^{11}/g depending on the examinees' age and sex, which is in agreement with genetic and cultural findings in relation to both the total number of microorganisms and the dominant role of the bacteria of the genera *Eubacterium, Bacteroides, Clostridium,* and *Bifidobacterium* in the feces.

Key words: *Fatty acids, meconium, microorganism, fecal microbiota*

Introduction

Conventional methods for determining intestinal microbiota composition rely on the cultivation of bacteria on selective media. However, many bacteria are difficult to culture or are uncultivable and often media are not truly specific or are too selective for certain bacteria. Molecular tools introduced in microbial ecology have made it possible to study the composition of intestinal flora in a culture-independent way based on the detection of rRNA (1).

The microbial detection technique using fatty acid (FA) markers is similar to the genetic one (polymerase chain reaction (PCR), 16S RNA sequence measurements, etc.) since the composition of FAs was determined in DNA and reproduced via replication of a genome portion by transfer RNA, followed by mitochondrial FA synthesis by messenger RNA. Therefore, the profile of FA bacteria is their business card or 'fingerprint' (2). It is as conservative as the structure of DNA, but it is also prone to mutations due to environmental factors. The stability of a set of the FAs that form microbial cells, as evidenced by bacteriological paleontological studies that show that FA composition of some microbes and the pool of their FAs as a whole, has remained constant for as long as 2.5 billion years (3).

Chemical methods for microbial differentiation are finding increasing use and they are frequently more rapid and universal than earlier approaches (4). For these purposes, gas chromatography (GC) and GC in combination with mass spectrometry (GC-MS) are in most common use, and provide unique information on the composition of monomer chemical components of a microbial cell and metabolites (5–7). Markers of this type may be determined and used to detect microorganisms in a complex environment matrix (8–11). Different applications of chemical marker analysis have been described (12,13). Chemo differentiation is widely employed as a method for identifying and confirming the taxonomic position of microorganisms. The technique is used to work with microorganisms isolated in pure cultures and based on the application of very large databases containing information on the composition of FAs of several thousands of bacterial strains and microscopic fungi. This system is exemplified by a specialized Microbial Identification System

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chromatograph ('Sherlock'; MIDI Inc., Newark, DE, USA (14). The specific features of the composition of FA are used along with other parameters in bacterial taxonomy and clinical bacterial diagnosis (15).

The capacities of GC-MS detection of microorganisms by their markers, including FAs, in practical medicine have been little studied. Attempts to detect bacteria from blood by using muramic (16–18) and β-hydroxymyristic (19) acids as a markers have been reported. Control of meningococci from the presence of β-hydroxylauric acid in blood (20) and gonococci from the concomitant presence of β-hydroxylauric and β-hydroxymyristic acids (21) have been proposed. GC-MS potential for diagnosis and studies on in clinical microbiology research have been discussed (5). Recently 3-hydroxy FAs in tissues (22) and saliva (23) were investigated as diagnostic markers of endotoxin intraperitoneal injection and chronic periodontitis.

We have previously described examples of microbial detection in infectious processes by GC-MS (24). Homeostasis of small molecules of microbial origin has been found in human blood, which is impaired in inflammation (25).

The microbiota of intestine is extremely rarely studied in gastroenterology, mainly during surgical intervention. If so doing, the limited range of nosocomial pathogens is mainly detected by cultural and biochemical methods under aerobic cultivation. That is why changes in the normal intestinal microbiota are more frequently determined from the composition of fecal microorganisms (26), by using, among other techniques, GC of long chain FAs (27). However, the chromatographic techniques used to detect a signal from flame ionization fail to provide adequate data on microbial markers due to the limited sensitivity, selectivity, and specificity of the technique in detecting a wide FA range required for analyzing such a complex microbial community as the intestinal microbiota.

This study has attempted to fill this gap by measuring the quantitative and qualitative composition of the microbiota of feces (as the reference substrate) from the perspective of the intestinal wall (together with a mucosal layer) in healthy donors and in patients with irritable bowel syndrome (IBS) and associated antibiotic diarrhea (AAD). Our first measurement of intestine mucosal microbiota was published in Russian (28). The present paper is an advanced version of that investigation and includes additional markers – fatty aldehydes – for monitoring intestinal dominants: *Eubacterium, Propionibacterium, Bifidobacterium, Closyridium*, and other plasmalogen-containing microbes. The concentrations of microbial FAs and aldehydes were measured by GC-MS in the mass-fragmentography mode, i.e. monitoring the specific ions of the mass spectrum of target substances (markers).

Material and methods

GC-MS

The averaged feces samples, 3–4 mg, were exposed to acid methanolysis in 1 M HCl in methanol at 80°C for 1 h. As a result of methanolysis, FAs were released as methyl esters. They were extracted twice with 200 μl of hexane, dried, and treated in 20 μl of N,O-bis(trimethylsilyl)-trifluoroacetamide at 80°C for 15 min to produce trimethylsilyl esters of hydroxy acids and sterols. A mixture of the esters in an amount of 2 μl was injected into the HP-5973 Hewlett-Packard GC-MS system (USA). The standard programs of the device were used to control and process data. The specimen was chromatographically separated on a capillary column with the methylsilicone chemically bonded phase HP-5ms Hewlett-Packard. The length of the column was 25 m; its internal diameter was 0.25 mm. The mode of an analysis was programmed; the rate of oven heating was 5°/min in the range of 130–320°C. The mass spectrometer was quadrupole with electron (70 Ev) ionization, it operated in the selective ion mode (SIM), regularly detecting 20 ions in each of 5 intervals. The intervals and ions were chosen so that the concentrations of marker FAs of detectable microbial species were selectively measured. The strong ion m/z = 87 in the spectra of FAs was used to detect minor quantities of microbial acids of C12–C20. Ion 75 was used to detect fatty aldehyde as dimethyacetals (DMA). Ion 175 was constantly taken in each interval to detect β-hydroxy acids, which it is specific to and intensive in the spectrum. Intensive ions 301, 315, and further every 14 unit masses (of structure M-15) were chosen as witnesses of the molecular ion of hydroxy acids (tridecanoic, tetradecanoic, and the following acids in the homologous series). Ion 312 was taken as molecular to reveal the isomers of nonadecanoic acid, which are of importance in diagnosing staphylococci and enterococci. A total of 33 ions were entered into a monitoring program for SIM recording of a chromatogram. According to our estimates, this algorithm for detecting the mass spectral parameters of a biological specimen can detect about 200 known microbial FAs, alcohols, DMAs, and sterols, which is sufficient to reveal and assay more than 170 taxons of clinically significant microorganisms at the level of a genus or a species.

The preset program was applied to automatically integrate the peak areas of markers on the mass fragmentograms. Then these data were entered into the calculation program prepared in the EXCEL tables. The data were calibrated with deuterated tridecainoic acid as internal standard and elsewhere published data on FA composition of clinically important microorganisms and our clinical isolates as well. Biological

reproducibility (accuracy of measurements) was calculated as 20% in statistical measurements (24,25).

Calculation of the composition and number of effective microbial cells

While drawing up a program of analysis, developing an algorithm for identifying and calculating the number of microorganisms, we were guided by the principle of a limited number of the members of a local microbial community, the recognition of the profile of microbial FAs as an image during computer-assisted identification, and by the principle of a marker in the application only to the microbial community under consideration (10).

Different microorganisms are known to contain about 200 FAs as components of cellular wall lipids, which distinguish them from human cells. In intestinal microorganisms some substances appeared to be specific to one taxon. The number of cells of these microorganisms was calculated by the concentration of a marker substance, by using the known data on the content of FA in the microbial cell, on the conditions for preparing a sample and calibrating a device.

The chromatographic peak area of a marker is in proportion to its concentration and thus to the concentration of a respective microorganism, which is estimated as the number of cells N_1 per unit of volume or weight of a sample, using the formula:

$$N_1 = Ai[Mst/(q^*Msam^*Ast)]/Ri_1$$

where the expression enclosed in brackets, the constant coefficient

$$\kappa = Mst/q/Msam/Ast = Mst(mg)/5.1 \times 10^{(-15)}g/Msam(mg)/Ast$$

In these formulae, Ai is the peak area of a marker, Mst is the amount (mg) of the reference injected into the sample, Msam is the amount of the sample, Ast is the peak area of the reference, Ri_1 is the proportion (%) of the marker with the index i in the profile of FA of a detected microbe with number 1 (N_1), q is the coefficient equal to $5.1 \times 10^{(-15)}$g, which contains the basic value calculated with reference to the number 5.9×10^{12} microbial cells available in 1 g of microbial biomass and the proportion of cellular FA, which is taken as, on the average, 3%.

The number of cells of any following microorganism may be calculated, by using the similar formula $N_2 = Ai \times k/Ri_2$ and so on, by multiplying the peak area of a marker (Ai) that is used to make calculations, by the coefficient k and dividing by the proportion of the marker as a part of total FA of this microorganism as a percentage.

The effective number (i.e. corresponding to the marker's concentration measured at this moment) of bacteria including *Clostridium ramosum* group, *C. perfringens*, *C. difficile*, *Bacteroides fragilis*, the yeast *Candida albicans*, bacteria of the genera *Bifidobacterium*, *Eubacterium*, *Propionibacterium*, *Streptomyces*, *Nocardia*, *Alcaligenes*, *Pseudomonas*, *Enterococcus*, *Staphylococcus*, the family *Enterobacteriaceae*, and others, as well as non-specific fungi from ergosterol (*Aspergillus*, *Mucor*, etc.) was estimated by the same procedure – one taxon, one marker. Some viruses are involved in the synthesis of sterols in human cells, which can be determined from the product of transformation by their enzyme of human cholesterol to cholestendiol (herpes viruses), isomers of cholestadiene and cholestadienon (cytomegaloviruses). If the substance does not have the property of a marker, i.e. it may be referred to two taxons or more, here the contribution of each microorganism, if the solution of a system of equations for two substances (or, accordingly, more) is applied. We have described the procedure for calculating ecological microbial communities elsewhere (3,10,29).

Detection of taxonomic FA

By using the known data on the most common intestinal microorganisms (30) we formed a local database on the composition of FAs (Table I, database), which was used to identify markers and to make calculation formulae. Below is shown the substantiation of affiliation of markers to specific microorganisms that are putative intestinal wall inhabitants, as well as the choice of a specific marker or a scheme for calculation of their concentrations.

Bifidobacterium. The number of bifidobacteria was estimated from the component of the specific cellular membrane lipid plasmologen, wherein one of the Fas of glyceride is replaced by fatty aldehyde. The distinctive feature of bifidobacteria is 9-octadecenoic aldehyde (31) that was registered as a measure of their concentrations.

Lactobacillus. Lactobacilli have clear markers, such as lactobacillic acid (31) and cis-vaccenic acid (18:1Δ11), which are also found in other bacteria, such as *Pseudomonas* and *Enterobacteriaceae*. However, *Pseudomonas* is rarely detectable in the intestine in noticeable concentrations and the cross determination with enterobacteria is taken into account in the equation of balance of FA concentrations, by using additional markers. Cis-vaccenic acid was used here to control lactobacilli.

Enterobacteriaceae family. Enterobacteriaceae are close in the profile of FA, at the same time their

Table I. List of fatty acids, aldehydes, and sterols detected in the feces indicating the most likely microorganisms in whose cells they usually were found.

No.	Abbreviation*	Chemical name	Most likely microorganisms
1	C10	Decanoic	*Streptococcus*
2	C12:0	Dodecanoic	Non-specific
3	iC13	*iso*-Ridecanoic	*Bacillus subtilis*
4	a13	*anteiso*-Tridecanoic	*Bacillus cereus*
5	i14	*iso*-Tetradecanoic	*Peptostreptococcus anaerobius, Streptomyces, Bacillus, Bacteroides*
6	14:1ω7	7,8-Tetradecenoic	*Haemophilus parahaemolyticus, Arcobacter*
7	14:1ω5	9,10-Tetradecenoic	*Clostridium*
8	14:1ω3	11,12-Tetradecenoic	*Nocardia*
9	10Me14	10-Methyl-tetradecanoic	Actinomycetes
10	15:1ω6	9,10-Pentadecanoic	*Clostridium*
11	i15	*iso*-Pentadecanoic	*Propionibacterium*
12	a15	*anteiso*-Pentadecanoic	*Staphylococcus, Bacillus*
13	10Me15	10-Methyl-pentadecanoic	*Actinomycetales*
14	i16:1	*iso*-Hexadecenoic	*Pseudonocardia*
15	16:1ω9	7,8-Hexadecenoic	*Clostridium ramosum*
16	16:1ω5	11,12-Hexadecenoic	*Ruminococcus*
17	i16:0	*iso*-Hexadecanoic	*Streptomyces, Corynebacterium betae, Curtobacterium, Cellulomonas*
18	16:0	Hexadecanoic	Non-specific
19	10Me16	10-Methyl-hexadecanoic	*Rhodococcus*
20	i17:1 I	*iso*-Heptadecenoic I	*Campylobacter mucosalis*
21	17:1	Heptadecenoic	*Candida albicans, Mycobacterium*
22	i17:0	*iso*-Heptadecanoic	*Bacillus, Prevotella, Propionibacterium*
23	a17:0	*anteiso*-Heptadecanoic	*Corynebacterium* CDC group
24	17cyc	*cyclo*-Heptadecanoic	*Enterobacteriaceae*
25	17:0	Heptadecanoic	Non-specific
26	10Me17	10-Methyl-heptadecanoic	*Actinomadura*
27	18:1ω7	11,12-Octadecenoic	*Lactobacillus*
28	i18	*iso*-Octadecanoic	*Clostridium difficile, Bacillus subtilis,*
29	10Me18	10-Methyl-octadecanoic	*Mycobacterium, Nocardia, Corynebacterium bovis, C. xerosis* group, *C. urealyticum, Actinomycetes*
30	19cyc	*cyclo*-Nonadecanoic	*Enterococcus,*
31	20:1	Eicosenoic	*Propionibacterium jensenii, Actinomyces*
32	i19	*iso*-Nonadecanoic	*Bacillus subtilis, Bacteroides hypermegas*
33	a19	*anteiso*-Heptadecanoic	*Staphylococcus*
		Hydroxy-acids	
34	h10	Hydroxy-decanoic†	*Pseudomonas, Comamonas*
35	hi11	Hydroxy-iso-undecanoic	*Stenotrophomonas maltophilia*
36	h12	Hydroxy-lauric	*Acinetobacter, Moraxella*
37	hi13	Hydroxy-iso-tridecanoic	*Stenotrophomonas maltophilia*
38	h13	Hydroxy-tridecanoic	*Selenomonas*
39	h14	Hydroxy-myristic	*Fusobacterium, Haemophilus*
40	hi15	Hydroxy-*iso*-pentadecanoic	*Prevotella, Flavobacterium, Capnocytophaga*
41	h16	Hydroxy-palmitic	*Bacteroides, Wolinella*
42	hi17	Hydroxy-*iso*-heptadecanoic	*Bacteroides fragilis*
43	h17	Hydroxy-heptadecanoic	*Bacteroides ruminicola*
44	10h18:1	10-Hydroxy-octadecenoic	*Clostridium perfringens*
45	h18	Hydroxy-stearic	*Helicobacter pylori*
46	10h18	10-Hydroxy-stearic	*Clostridium perfringens*
47	ha15	Hydroxy-*anteiso*-pentadecanoic	*Bacteroides*
48	ha17	Hydroxy-*anteiso*-heptadecanoic	*Bacteroides*
49	2h12	2-Hydroxy-lauric	*Pseudomonas aeruginosa, Alcaligenes*
50	2h14	2-Hydroxy-myristic	*Sphingomonas*
51	2hi15	2-Hydroxy-*iso*-pentadecanoic	*Flavobacterium, Flexibacter*
52	2h16	2-Hydroxy-palmitic	*Pseudomonas cepacea, Flexibacter*
53	2hi17	2-Hydroxy-*iso*-heptadecanoic	*Flexibacter*
	Fatty aldehydes		
54	14a	Tetradecanoic	*Eubacterium lentum, Bifidobacterium*
55	i15a	*iso*-Pentadecanoic	*Propionibacterium acnes*
56	a15a	*anteiso*-Pentadecanoic	*Eubacterium, Peptostreptococcus*
57	15a	Pentadecanoic	
58	16:1a	Hexadecenoic	*Eubacterium moniliforme*
59	i16a	*iso*-Hexadecanoic	*Eubacterium lentum*

(Continued)

Table I. (*Continued*)

No.	Abbreviation*	Chemical name	Most likely microorganisms
60	16a	Hexadecanoic	Non-specific
61	17a	Heptadecanoic	*Propionibacterium freudenreichii*
62	i17a	*iso*-Heptadecanoic	*Propionibacterium*
63	a17a	*anteiso*-Heptadecanoic	*Eubacterium lentum*
64	18:1ω9a	9,10-Octadecenoic	*Bifidobacterium*
65	18:1ω7a	11,12-Octadecenoic	*Eubacterium, Clostridium ramosum*
66	19cyc a	*cyclo*-Nonadecanoic	*Enterococcus*
	Sterols		
67	Coprostanol	Dehydro-cholesterol	*Eubacterium*
68	Cholestendiol		Herpes virus
69	Campesterol		Microscopic fungi
70	β-Sitosterol		Microscopic fungi
71	Cholestadienon		Cytomegalovirus
72	Ergosterol		Microscopic fungi

*In 17:1, 17 is the number of carbon atoms, the figure after the colon denotes the number of double bonds; h, hydroxy-acid; a,I, indicates methyl-branching; cyc, cyclopropanoic acid. For example, 2hi15 means 2-hydroxy-*iso*-pentadecanoic acid.
†3-Hydroxy-acids, if position of hydroxyl is not indicated.

genus-specific, occasionally species-specific, differentiation in the pure culture of cells is assumed, but they are barely distinguishable if they are simultaneously present in the community of microorganisms. Their markers are β-hydroxymyristic acid (h14), cycloheptadecanoic (17cyc), and cic-vaccenic acids have a rank of a family, with multiple intersections with the representative of other families. Here in the absence of *Pseudomonas*, the number of enterobacteriaceae as a whole can be actually measured by the concentration of 17cyc (15,31).

Eubacterium. The representatives of this genus comprise one of the basic intestinal inhabitants. Their marker, dehydrocholesterol (coprostanol), is a product of the interactive metabolism of *Eubacterium* and cells of the host's body (32). For differentiation of eubacteria at the level of a species and subspecies, their differences in the composition of fatty aldehydes (31) are used (see Table IV). Furthermore, the species of *Eubacterium* have been determined by specific fatty aldehydes, among them, *E. lentum* by iso-hexadecanoic aldehyde (i16a), a group of strains of this species, including *E. lentum* 7741, and others wherein tetradecanoic aldehyde (14a) are the leaders (33). The main group of species of the genus *Eubacterium* (*E. moniliforme*, *E. nodatum*, *E. sabureum*, and others) were separately determined by 11-octadecenoic (18:1ω7a) aldehyde (31).

Propionibacterium. Iso- and anteiso-heptadecanoic (i17a and a17a) aldehyde were assigned to this genera according to 'Sherlock' (31) and our own measurements of *Propionibacterium freudenreichii* type culture from the All-Russian Collection of Microorganisms – VKM.

Peptostreptococcus anaerobius. This microorganism is known to have less common even iso-acids with a number of carbon atoms from 10 to 16 in the profile of FAs (34). We used iso-dodecanoic (i12) and iso-tetradecanoic (i14) acids as markers.

Ruminococcus. These were determined by 11- hexadecanoic acid found by use in the natural isolates of ruminococci (35).

Propionobacterium acnes. For identification of this microorganism, iso-pentadecanoic aldehyde (i15a) (our own data) minus the contribution of *Eubacterium* spp. was used.

Bacillus. Bacilli of the species *B. cereus* and *B. subtilis* may be detected by specific branched acids with 13 carbon atoms: i13 and a13. *B. megaterium* was determined from the residue of anteiso-pentadecanoic (ai15) acid, using the balance equation (36).

Acinetobacter. It is convenient to use 2-hydroxy dodecanoic acid (15) as a generic sign in the presence of 3-hydroxydodecanoic acid.

Clostridium perfringens. They have clear markers that are characteristic of the clostridia group including, besides *C. perfringens*, *C. histolyticum*, and *C. oedematiens*. These are 10-hydroxystearic and 10-hydroxy-octadecenoic (10h18) acids readily detectable by specific ions in the mass spectrum [our data, published in Russian]. These substances are not cellular components of clostridia themselves, but they are associated with the breakdown of tissue cells of the organism by bacterial enzymes (37).

C. difficile and other clostridia. *C. difficile* differs from other clostridia in that iso-octadecanoic (i18) acid

(38) is in the composition of FA. *C. histolyticum* differs in that iso-tetradecenoic acid is present in FA profile, and the *C. ramosum* group differs in that 7-hexadecenoic (16:1Δ7) acid (31) is in the FA profile.

Bacteroides. The anaerobic bacteria of the group *B. fragilis* have a good marker – a pair of branched hydroxy acids: hydroxy-iso-heptadecanoic (hi17) and anteiso-heptadecanoic (ha17) ones (39). The number of the remaining bacteroides was estimated from the residue in the balance of hydroxy-hexadecanoic acid.

Streptococcus. Many streptococci are 'invisible' in the presence of biological fluid components due to the coincidence of intrinsic FAs with the acids of a substrate. However, a group of oral or α-streptococci, such as *Strep. mutans*, *Strep. salivarius*, etc. is detectable from decanoic acid C10 (40) and the monounsaturated acids 16:1Δ7 and 18:1Δ11 (31). *Strep. mutans* was detected by its specific 11-eicosenoic acid – 20:1ω9 (31).

Enterococcus. Enterococcus faecalis and *E. faecium* with their prevalence in the community may be also be detectable by cis-9,10-methylene-hexadecanoic acid (9,10-19cyc) acid (31).

Candida albicans, Mycobacterium. Heptadecenoic acid is a specific sign of the yeast *C. albicans* in the lipid fraction of human biological fluids [our own data]. It cannot be ruled out that it may be also referred to mycobacteria (41).

Microscopic fungi. The non-specific marker of clinically significant microscopic fungi (*Aspergillus*, *Candida*, *Mucor*, etc.) is ergosterol (42), as well as campesterol and β-sitosterol (43) [authors' own measurements].

Flavobacterium (Sphingobacterium, Chriseobacterium). These bacteria have branched odd 2-hydroxy acids in the composition of cellular sphingolipids (2hi15, 2hi17) that may serve as markers in clinical tests (15).

Streptomyces, Nocardiopsis. The FA profiles of biological fluids from patients contain a great amount of iso-hexadecanoic acid (i16), which substantially exceeds the possible proportion of *P. anaerobius* and *Bacillus* that have this substance as a constituent of the cellular membrane. The representatives of the genus *Streptomyces* and some other actinomycetes (such as *Nocardiopsis dasonvilley* isolated by us from an intestinal mucosal biopsy specimen) are rare organisms (of those we know) that have this sign. The strains of streptomycetes, which have as high as 40% of i16 in the profile of FAs have been described in the literature (44). There is also evidence for the participation of streptomycetes in the colonization and inflammation of different human organs.

Actinomadura. They were determined by 10-methyl-heptadecanoic acid (10Me17) (44) minus the contribution of rhodococci.

Pseudonocardia. This genus determined by iso-hexadecenoic acid (i16:1) (44) minus the contribution of rhodococci to it's measured value.

Rhodococcus. Rhodococci are likely to be responsible for the presence of 10-methyl-hexadecanoic acid (10Me16) (44) in the composition of FAs of all the samples we have studied.

Nocardia. They were determined by the tetradecenoic (presumably 14:1d11) acid isomer detected by us in the isolate of *Nocardia* from the patient's blood. *Nocardia asteroides* and others were detected by trans-9,10-hexadecenoic acid (16:1ω7t) (44).

Helicobacter pylori. Hydroxyoctadecanoic acid (h18) is usually present in the clinical samples. This hydroxy acid is characteristic of the genus *Francisella* and the species *Helicobacter pylori* (45). In our case it is logical to assign the presence of h18 to *H. pylori* or *F. filomiragia*. *H. pylori* is usually associated with chronic gastritis; however, it is detectable in the tissues of patients with oral aphthous ulcers (46), atherosclerotic plaques (47), and hepatic abscesses. Moreover, GC-MS analysis of the strains isolated from the intestinal biopsy specimens in this study indicated the presence of 11Me18:1, 2h18:1, 11-OMe-19, and cholesterol along with H. *pylori*-characteristic FA (18:1d11, 16:0, 19cyc, 3h16, 3h18). In the manual of non-fermenting gram-negative bacteria (15), the FA profile of *H. pylori* showed 11Me18:1 (under the name of 19:1br).

Campylobacter mucosalis. This species has rare iso-heptadecenoic (i17:1) acid (31) as a component of FA, which was monitored by taking into account the contribution of bacteria of the genera *Chriseobacterium* and *Flavobacterium*, if their marker 2hi15 was present.

Fusobacterium. The only distinctive component in the cellular FA of these bacteria is 3-hydroxymyristic acid (3h14) (48), which also occurs in other clinically significant gram-negative microorganisms, such as *E. coli*, *Alcaligenes*, *Serratia*, etc. Therefore, *Fusobacterium* and *Haemophilus* may be determined only from the residue, using the balance equation for 3h14.

Staphylococcus. Staphylococci are known to contain odd iso- and anteiso-branched acids with the number

of carbon atoms 15, 17, and 19 (49). Anteiso-nonadecanoic (ai19) acid may be used as a generic marker in this community.

Corynebacterium CDC groups. A residue is frequently observed after consideration of all the microorganisms that have anteiso-heptadecanoic acid in the composition of FA. This may be most likely to be attributable to *C. betae*, *C. aquaticum*, *Listeria*, and *Brevibacterium*, as well as individual groups of CDC A-3, A-4, A-5, B-1a, B-3a, B-1b, and B3b, which contain particularly high levels of the acids a15 и a17 [50].

Viruses. By comparing our data with the results of examination of patients with different diseases using immunological and genetic studies, we found a correlation in the appearance of the cholesterol metabolite cholestenediol in herpes virus infection and cholestadienone in cytomegalovirus infection.

The feces specimens were examined for the content of 135 microbial substances, which provide information on more than 170 taxons of microorganisms. All specific substances were confirmed just in the objects studied using mass spectra in the direct scanning while comparing them with the standard libraries (NIST and Wiley) of the mass spectra of the GC-MS system.

Results

GC-MS analysis of FA fractions in feces specimens revealed that the major components (at the level of $>$ 1% relative content) are even acids with 12–18 carbon atoms: C18:1, C16:0, C18:2, C18:0, C16:1 (in order of decreasing levels in the profile of FAs), as well as polyunsaturated FAs (C20:n, C22:n), cholesterol, aldehydes, and 2-hydroxy acids. The level of long-chain acids (C23 and higher) is occasionally $>$ 1%. Each of the odd acids C15:0 and C17:0 forms about 1%.

The above substances are the lipid components of human cells and form the natural background against which the minor microbial components that are not characteristic of the human cells are detected in the examined samples. The chromatograms obtained by the selective ion method can confidently detect microbial components in the presence of predominant human waste components. For the most part, the peaks of target substances are superposition-free, rather distant from the peak of a substrate and therefore they may be accessible for automatic random integration in accordance with the standard program of the GC-MS system. The list of FAs, aldehydes, and sterols detected in feces specimens is given in Table I, indicating the most likely microorganisms in whose cells they usually were found.

The composition of intestinal microbiota has not been previously studied by GC-MS analysis. Moreover, there are no values of the composition of its microorganisms, measured by any routine procedure. In doing so, we cannot compare our results with the reference values. Therefore we also had to measure the microflora of feces in some samples from healthy persons of different ages and sex, as well as those of neonatal transitional stool and meconium (Table II). Since it had been thoroughly studied in the qualitative and quantitative senses, we used the fecal microflora as a reference material to substantiate the validity of the data of the future analysis of the microbiota of the intestinal wall and other objects by MS of microbial markers.

The whole number of microorganisms in feces varies from 3×10^{10} to 4×10^{11} in healthy adults and children. Minor values are specific for children and the elderly, which are consistent with known data. Really, the number of bifidobacteria is maximum in children and adults in comparison with newborn and older persons (Table II). In Table III, the microorganisms are distributed by groups: the first entries show all anaerobes, with the leading ratio of *Eubacterium*. Next are clostridia, bacteroides, lactobacilli, and bifidobacteria. In persons with a formed intestinal microbiota, the proportion of anaerobes is 70–90% of the total number, as evidenced by our measurements. The aerobes are mainly represented by cocci and bacilli of different taxons (2.4–13.5%), as well as aerobic actinomycetes (actinobacteria) (1.1–15.3%) and microscopic fungi. Enterobacteria, pseudomonads, other gram-negative aerobes, and viruses are present in minor concentrations (0.1–7.3%).

Discussion

To confirm the reliability of these and subsequent measurements, we present their comparison with the known estimates obtained by cultural, biochemical, and genetic studies (Table IV) (51–53). The total number of microorganisms in the feces was in the range of 0.3–4 $\times$ 10^{11} cells/g, which agrees with the known literature measurements obtained by genetic and cultural and biochemical studies. The relative number of anaerobes, i.e. 70–90%, as shown by our data, is also in agreement with the known number. It is difficult to compare the genus-specific distribution in this study with that available in the literature as the distribution has a wide range of values – within 36 orders of magnitude. Nevertheless, our data coincide with those on the priority of the genus *Eubacterium*, which numbered 0.3–3 $\times$ 10^{10} cells/g (10^9–10^{12}, as shown by the data available in the literature), on the number of bacteroids, i.e. 0.2–2.4 $\times$ 10^{10} cells/g (10^{10}–10^{12} according to the known data), *Clostridium* 10^9–10^{11}cells/g (10^5–10^{11}, respectively), *Bifidobacte-*

Table II. Results of examination of the microbial composition (cell/g × 10^6) of feces from healthy persons aged 12–60 years, neonatal meconium, and transitional stool of newborn and IBS patients before and after treatment.

		Colonization level, cells/g × 10^6, mean value					
No.	Microorganism	Meconium (n = 2)	Newborn (n = 2)	Adults (n = 7)	Elderly (n = 1)	IBS before treatment (n = 5)	IBS after treatment (n = 3)
1	*Eubacterium lentum*	144	83	8213	2792	3223	978
2	*Eubacterium*	0	0	13 743	11 579	14 251	4693
3	*Propionibacterium*	18	0	13 442	7632	7875	4377
4	*Ruminicoccus*	315	368	506	30	1280	93
5	*Peptostreptococcus*	5519	539	2514	2991	6636	6384
6	*Clostridium histolyticum*	6	45	112	6	78	5
7	*Clostridium propionicum*	0	828	4055	384	829	198
8	*Clostridium ramosum*	638	884	3281	2096	1635	706
9	*Clostridium difficile*	1333	622	1046	820	390	377
10	*Clostridium perfringens*	6	34432	45470	9401	55209	11452
11	*Bacteroides hypermegas*	0	34	151	99	364	101
12	*Porphyromonas*	0	0	55	82	95	38
13	*Prevotella*	74	0	3137	8149	3452	2430
14	*Bacteroides fragilis*	0	0	1451	3223	2763	1575
15	*Bacteroides ruminicola*	52	0	2208	2130	6764	2822
16	*Fusobacterium*	0	601	385	428	907	280
17	*Bifidobacterium*	78	122	6589	1769	2441	2131
18	*Lactobacillus*	1054	2102	26 354	4545	19 070	3549
19	*Helicobacter pylori*	33	251	9968	368	2427	505
20	*Pseudomonas aeruginosa*	0	0	21	42	5	4
21	*Acinetobacter*	0	27	182	539	318	132
22	*Stenotrophomonas*	0	8	14	0	0	0
23	*Alcaligenes*	142	638	112	159	116	71
24	*Flavobacterium*	3	16	19	25	29	22
25	*Enterobacteriaceae* spp.	0	365	359	0	138	19
26	*Campylobacter*	125	14	44	0	9	30
27	*Bacillus cereus*	94	27	445	181	247	220
28	*Bacillus megaterium*	6	66	623	0	431	774
29	*Enterococcus*	10	1643	855	921	292	154
30	*Staphylococcus*	556	226	531	1991	271	175
31	*Streptococcus (oral)*	160	1169	4503	0	421	443
32	*Streptococcus mutans*	455	44	818	0	542	601
33	*Enterococcus faecalis*	0	0	1573	3587	1927	0
34	*Streptococcus intermedius*	0	685	2659	0	1371	2322
35	*Coryneform CDC group*	0	306	521	0	221	172
36	*Nocardia* spp.	54	18	146	70	172	86
37	*Actinomycetes 10Me15*	69	42	48	34	78	61
38	*Pseudonocardia*	133	41	43	477	128	153
39	*Streptomyces*	828	164	2378	518	1420	900
40	*Rhodococcus*	414	283	395	74	140	166
41	*Mycobacterium/Candida*	41	331	771	65	140	32
42	*Actinomadura*	9	0	22	0	7	11
43	*Nocardia asteroides*	235	126	868	0	159	56
44	*Actinomycetes 10Me14*	155	472	167	54	425	155
45	Microscopic fungi 1[a]	160	5350	2462	1173	783	500
46	Microscopic fungi 2[b]	290	6240	5118	8	1958	1526
47	Cytomegalovirus	16	0	59	0	29	19
48	Herpes virus	204	68	20	13	54	209
	Total	13435	59292	168490	68484	141569	51770

Gas chromatography-mass spectrometry of microbial markers was used. Accuracy of measurements is 20% reproducible.
[a]Microscopic fungi producing campesterol.
[b]Microscopic fungi producing sitosterol.

rium 10^8–10^{10} cells/g (10^{10}–10^{12}). These results confirm that GC-MS analysis of the fecal microbiota yields valid data on their number. Therefore, any data on the composition of microorganisms in biopsy specimens or any other clinical, as well as environment samples, may also be considered valid.

Table III. Grouping of microorganisms (cell/g × 10^6) of feces from healthy persons aged 12–60 years, neonatal meconium, and transitional stool of newborn, and IBS patients before and after treatment.

	Colonization level, cells/g × 10^6, mean					
Microorganism group	Meconium (n = 2)	Newborn (n = 2)	Adults (n = 7)	Elderly (n = 1)	IBS before treatment (n = 5)	IBS after treatment (n = 3)
Total	13435	59292	168490	68484	141569	51770
Total anaerobes	9273	40918	142701	58544	129721	42734
Anaerobes, %	69	65	79	85	90	84
Aerobic gram-negative bacteria	271	1068	752	765	616	278
Cocci and bacilli	1281	3861	12 007	6680	5502	4690
Actinobacteria	1938	1783	5361	1292	2890	1793
Fungi	450	11 591	7579	1181	2741	2026
Viruses	220	68	80	13	83	228
Bacteroides	126	34	7002	13683	13439	6965
Clostridium	1982	36810	53964	12707	58140	12738
Proportion, %						
Aerobic gram-negative	1.9	4.4	0.5	1.1	0.6	0.5
Cocci and bacilli	9.3	9.8	7.2	9.8	4.7	7.3
Actinobacteria	14.3	7.8	3.5	1.9	2.4	3.2
Fungi	3.5	12.3	9.4	1.7	2.6	4.9
Viruses	1.6	0.3	0.1	0.02	0.1	0.5
Enterobacteria	0.0	2.5	0.2	0.0	0.1	0.1
Bifidobacteria	0.5	0.2	4.6	2.6	2.4	3.9
Lactobacilli	8.0	17.1	13.6	6.6	10.7	5.9
Clostridium, %	14	39	25	18.6	29	25

Accuracy of measurement is 20% reproducible.

Different investigations show that the fecal microbiota contains bifidobacteria almost 100 to 0.1% (Table III). The range of three orders of magnitude is unlikely to be due to the fact that human beings are different. Each study presents serious statistical data and a conscientious analytical procedure. The difference is more likely to be considered as the specific features of the comparable methods for measurements. Without going into details, it may be concluded that the effect of a predominance of *Bifidobacterium* is produced by the routine procedure for analyzing only *Bifidobacterium* and opportunistic microorganisms in studying disbiosis. *Eubacterium*, bacteroids, and *Clostridium*, which are at least several times more than *Bifidobacterium* are seen to be out of the view of a microbiologist. This delusion looks natural if we recall that it is practice to consider within general microbiology that not more than 20% of the microorganisms in any habitat are on the average cultured in the microbial community. Molecular genetic studies indicate that 60–80% of the fecal microbiocenosis cannot be determined by cultural techniques. Mass spectrometric data correlate with genetic data (within the comparability of microbiological estimates) and equally demonstrate that *Eubacterium*, bacteroids, and *Clostridium* alone and in combination are an order of magnitude higher than *Bifidobacterium*.

MS made it possible to measure the numbers of more than 50 taxons of intestinal microorganisms in the feces. These data show that *Eubacterium* spp. are prevalent. The affinity of *Eubacterium* for *Clostridium* should be noted. The ninth edition of Bergey's manual (54) directly reads that the genus *Eubacterium* has been designed for convenience so that it should include weakly spore-forming clostridia. If the heterogenicity of both genera, which is still known and unordered so far, is mentioned, it can be seen that the intestinal microbiota is a predominant continuum of strains and species of the genera *Clostridium* and *Eubacterium* in their present arrangement with the equivalent total amount of propionibacteria, bacteroids, bifidobacteria, and lactobacilli. The proportion of another biological diversity of intestinal microorganisms (as evidenced by MS) is as high as 10% in feces. The fact that the genera *Clostridium* and *Eubacterium* are closely genetically related is suggested by the current absence of specific probes for each genus. The probes designed for *Clostridium* determine *Eubacterium* in a cross-reaction and vice versa. Thus, in addition to *Eubacterium*, the probe proposed to determine a group of new clostridia headed by *C. coccoides* (intestinally detected in 1997) also includes ruminococci in this group.

The presented data suggest that the genus *Eubacterium* is of importance in the formation and functioning of the intestinal microbiota and we tend to consider them to be a digestively important group of peptolytic and cellulolytic organisms. The fundamental important feature of the representatives of the genus *Eubacterium* is noteworthy, i.e. the capacity to

Table IV. Comparison of data for analysis of fecal microbiota by genetic, cultural and biochemical, and mass-spectrometric studies.

Parameter	Composition of adult fecal microbiota, cells/g wet weight			
	Mass spectrometry (this paper)	Genetic study, Harmsen et al. (51)	Cultural study	
			Schaechter et al. (52)	Wittmann (dry weight) (53)
Total number	$0.3–4 \times 10^{11}$	2.1×10^{11}	$10^{10}–10^{12}$	2×10^{11}
Proportion of anaerobes, %	70–90	Up to 100	Up to 100	33–100
Eubacterium	$0.3–3 \times 10^{10}$	1.4×10^{10}	$10^{9}–10^{12}$	3×10^{10}
Bacteroides	$0.2–2.4 \times 10^{10}$	3.6×10^{10}	$10^{10}–10^{12}$	10^{11}
Clostridium	$10^{9}–10^{11}$	2.9×10^{10}	$10^{5}–10^{11}$	3×10^{10}
Bifidobacterium	$10^{8}–10^{10}$	6×10^{9}	$10^{8}–10^{12}$	2×10^{8}

produce hydrogen. This is a key property of the microbial consortium that effects the digestion of an organic substrate during anaerobic processes in nature (in the marsh), in the rumen, and in biotechnology during the anaerobic fermentation of all kinds of waste products and the production of a biogas. The human intestinal mucosa is essentially a similar bioreactor. Methane is formed there and hence methanogenic archaebacteria, whose effectiveness strictly depends on the concentration of hydrogen in the system, are at work. In the methanogenic community, hydrogen-producing bacteria play a key regulatory role due to the feedback in the production and consumption of hydrogen in the primary process of carbohydrate cleavage to give rise to acetate.

There is no question that detection of a considerable amount of aerobic actinomycetes is an unexpected result. The specificity of their markers – branched FAs with a methyl group in the position Δ10 – gives no way of assuming some other taxonomic groups of microorganisms, other than the representatives of actinomycetales whose cell walls contain mycolic acids that are a source of 10Me-branched FAs. They are present in *Mycobacterium, Nocardia, Rhodococcus, Actinomadura* spp., and other actinomycetes, but they have not been found in higher organisms (fungi, plants, and animals). The presence of these molecules in human feces is supported by mass spectra in the chromatographic peak and by relative retention time, as well as by their analysis in the composition of type cultures of respective microorganisms. The bacteria of the genera *Streptomyces* and *Nocardiopsis* are also confirmed by the unique marker iso-hexadecanoic acid (i16). Further, the profile of the branched FAs specific for streptomycetes was detected in the blood of septic patients in our practice (25). The list of actinobacteria should be extended by anaerobic actinomycetes and related microorganisms. These are *Propionibacterium*, *Actinomyces*, *Brevibacterium*, and corynebacteria. Finally, if we consider the fact that some manuals on microbiology, such as early ones (54), have assigned the genus *Bifidobacterium* to the family *Actinomycetaceae* so far, then it will turn out that actinomycetes are phylogenetically close to the traditionally known representatives of the intestinal parietal microbiota. With them, the intestinal microbiota grows in importance for the host, since actinomycetes are superior to all other microorganisms in producing antibiotics and vitamins and have a powerful enzymatic apparatus. The high intestinal colonization by actinomycetes does not look an unusual phenomenon if we bear in mind that they occur widely in the environment – soil, water, air, on the inner walls of dwelling and industrial premises (55). Their habitation in the human body looks natural under such circumstances. The guides on clinical microbiology mention that actinomycetes and related organisms, such as *Mycobacterium*, *Actinomadura*, *Propionibacterium*, *Actinomyces*, *Corynebacterium*, *Bifidobacterium*, are detectable in the human intestine and other organs. They (including *Bifidobacterium*) are known to be participants in infectious and inflammatory processes. Recent study of the composition of the human GI microbiota of 23 healthy adult subjects was performed on a pooled fecal bacterial DNA sample by combining genomic %G+C-based profiling and fractioning with 16S rRNA gene cloning and sequencing. The orders Coriobacteriales, Bifidobacteriales, and Actinomycetales constituted the 65 actinobacterial phylotypes (56). Earlier, nucleotide sequencing and amplification by PCR was done on the bacterial 16S ribosomal DNA present in a small bowel biopsy specimen taken from a patient with Whipple's disease. A search by computer for similar rRNA sequences filed in databases showed the Whipple's-associated organism to be most similar to bacteria in the genera *Rhodococcus*, *Streptomyces*, and *Arthrobacter*, and more weakly related to mycobacteria (57). However, the pathogenicity of actinomycetes, their antibiotic sensitivity, and treatment of their associated diseases is the subject matter of single specialized laboratories and clinics around the world. Difficulties in their bacterial diagnosis and cultivation are a handicap to the wide popularity of these microorganisms in clinical practice, for instance in multiple diseases associated with the altered intestinal microbiota (44).

Microbial markers have also been found in meconium from newborns. This confirms guesses and assumptions regarding prenatal intestinal colonization of the fetus. In fact, as reported some time ago (58), meconium dominant flora was composed of either enterobacteria or of streptococci. *Staphylococcus*, *Corynebacterium*, *Clostridium* (*C. perfringens*), *Bacteroides*, *Peptococcus* represented a very small proportion of the total flora. In the two children aged 48 h and more, the flora was more complex and *Bacteroides*, *Bifidobacterium*, *Veillonella*, *Peptostreptococcus*, *Clostridium*, and *Staphylococcus* were associated with streptococci and enterobacteria. A very small number of lactobacilli were found in one child only. Later, the presence of bacteria in meconium of 21 healthy neonates was investigated. The identified isolates belonged predominantly to the genera *Enterococcus* and *Staphylococcus* (59). We found similar features in meconium microbiota in our analyses, which are in good agreement with molecular and culturing methods, as can be seen from Table II.

The microbiota of IBS patients differs in genera composition from that of healthy adults and coincides with the data obtained by a genetic method (26). However, the GC-MS method has the advantage of measuring the number of each taxon. That is why we can monitor the dynamic of quantitative changes in microbiota. It is clear from Tables II and III that in spite of small differences in the total amount of microorganisms, proportion of anaerobes, numbers of aerobic gram-negative bacteria and clostridia (Table III), the genera and species composition differ substantially in healthy persons and IBS patients. For instance, numbers of *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *C. propionicum*, *Enterobacteriaceae* spp., *Streptococcus*, and microscopic fungi decreased, while the numbers of *Ruminicoccus*, *Peptostreptococcus*, *Bacteroides*, *Fusobacterium*, and *Acinetobacter* increased in patients as compared with healthy donors. We dare not speculate about the decrease in numbers of bacteria in feces after treatment. But we would like to admit that this is the same bacteria (*Ruminicoccus*, *Bacteroides*, *Fusobacterium*, *Acinetobacter*), which overgrows before treatment, as compared with healthy adults (Table II). We assume also, that the general microbiota deficiency is caused by reduction of microbes in the intestine after the termination of diarrhea.

The previous studies, which have relied almost exclusively on the use of culturing methods, have generated our current understanding of gut microbiology and ecology in infants. Gastrointestinal microbial ecology is experiencing a revival because of the development of molecular techniques, particularly techniques based on 16S rRNA genes, which are used to study complex bacterial ecosystems (60). So far, only a limited number of infant microbiota studies have been performed with molecular techniques, and very limited numbers of primers were used to characterize the diversity and quantity of fecal microbes. We demonstrate GC-MS as one more approach in studying microbiota in newborn and adults. This small number of experimental results could only demonstrate method and prove their compatibility with known ones both in genera composition in feces and tendency in age, health, and disease.

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EDITORIAL COMMENTS

G.A. Osipov, N.B. Boiko, N.F. Fedosova, S.A. Kasikhina and K.V. Lyadov: Comparative gas chromatography-mass spectrometry study of the composition of microbial chemical markers in feces

This article is a part of a very ambitious project, challenging the genetic way of determining the composition of a mixture of known and unknown microorganisms. Instead of studying microbial-derived genetic fingerprints the authors are studying lipid fingerprints in human fecal samples. Based upon an assumption that certain lipids are unique to certain species, the authors used gas chromatography-mass spectrometry technology for a qualitative and quantitative evaluation of lipids present in feces.

Both the reviewers are concerned about the specificity of the lipid markers in relation to specific microorganisms. As stated by one of the reviewers: 'The authors' statements that a certain microbe contains a certain lipid might be correct. The problem comes when they state that a certain lipid denotes a certain specific microbe. This is highly speculative.'

Surely, I am dealing with my reviewers' concerns. Why then accept a speculative paper with several weaknesses? Simply spoken: it represents a valuable approach to studying the human gut flora and its possible influence upon the human body. Again to simplify: neither microbial genes nor microbial names cause any harm to the body, but microbial products may do. Many lipids are very active biological compounds indeed. The presence of such compounds in the gut may have physiological and pathophysiological consequences for the host. That represents *Microbial Ecology in Health and Disease*!

Tore Midtvedt
Editor-in-Chief

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