https://doi.org/10.22416/1382-4376-2022-32-2-19-34

Direct and Indirect Methods for Studying Human Gut Microbiota

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Aim: To review the main methods of intestinal microbiota studying.
Key points. Currently, molecular genetic methods are used mainly for basic research and do not have a unified protocol for data analysis, which makes it difficult to implement them in clinical practice. Measurement of short chain fatty acids (SCFA) concentrations in plasma provides the data, which can serve as an indirect biomarker of the colonic microbiota composition. However, currently available evidence is insufficient to relate the obtained values (SCFA levels and ratio) to a particular disease with a high degree of certainty. Trimethylamine N-oxide (TMAO) levels in the blood plasma and urine can also reflect the presence of specific bacterial clusters containing genes Cut, CntA/CntB and YeaW/YeaX. Therefore, further studies are required to reveal possible correlations between certain disorders and such parameters as the composition of gut microbiota, dietary patterns and TMAO concentration. Gas biomarkers, i.e. hydrogen, methane and hydrogen sulphide, have been studied in more detail and are better understood as compared to other biomarkers of the gut microbiome composition and functionality. The main advantage of gas biomarkers is that they can be measured multiple times using non-invasive techniques. These measurements provide information on the relative proportion of hydrogenic (i.e. hydrogen producing) and hydrogenotrophic (i.e. methanogenic and sulfate-reducing) microorganisms. In its turn, this opens up the possibility of developing new approaches to correction of individual microbiota components.
Conclusions. Integration of the data obtained by gut microbiota studies at the genome, transcriptome and metabolome levels would allow a comprehensive analysis of microbial community function and its interaction with the human organism. This approach may increase our understanding of the pathogenesis of various diseases as well open up new opportunities for prevention and treatment.
Keywords: microbiota, microbiome, metabolome, transcriptome, sequencing, trimethylamine, trimethylaminoxide, short-chain fatty acids, hydrogen, methane, hydrogen sulfide
Conflict of interest: The authors declare no conflict of interest.

For citation: Ivashkin V.T., Medvedev O.S., Poluektova E.A., Kudryavtseva A.V., Bakhtogarimov I.R., Karchevskaya A.E. Direct and Indirect Methods for Studying Human Gut Microbiota. Russian Journal of Gastroenterology, Hepatology, Coloproctology. 2022;32(2):19–34. https://doi.org/10.22416/1382-4376-2022-32-2-19-34

Прямые и косвенные методы изучения микробиоты человека

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Цель публикации: рассмотреть основные методы исследования микробиоты желудочно-кишечного тракта.
Основные положения. В настоящее время молекулярно-генетические методы используются преимущественно для фундаментальных исследований и не имеют единого «протокола» анализа данных, что затрудняет их внедрение в клиническую практику. Исследование короткоцепочечных жирных кислот (КЦЖК) в плазме крови может служить косвенным маркером микробного состава толстой кишки, однако на сегодня нельзя с уверенностью связать количество и соотношение определяемых КЦЖК с определенной нозологической формой; изучение уровня ТМАО в плазме крови и моче также может отражать наличие в составе кишечной микробиоты особых кластеров бактерий, несущих гены Cut, CntA/CntB и YeaW/YeaX. Однако необходимы дальнейшие исследования по выявлению корреляционных связей между определенными заболеваниями, микробным составом ЖКТ, рационом и уровнем ТМАО. Газовые биомаркеры (водород, метан и сероводород) гораздо лучше изучены по сравнению с другими типами биомаркеров функции и состава микробиоты. Преимуществом газовых биомаркеров является возможность их неинвазивного, многократного измерения, что позволяет получать информацию о соотношении гидрогенных и гидрогенотрофных микроорганизмов.
Выводы. Объединение информации, полученной при исследованиях кишечной микробиоты на уровнях генома, транскриптома и метаболома, позволит произвести более глубокий анализ состава и функционирования микробиоты человека. Такой подход имеет несомненный потенциал для понимания патогенеза различных заболеваний и открывает возможности для разработки новых стратегий профилактики и лечения.
Ключевые слова: микробиота, микробиом, метаболом, транскриптом, секвенирование, триметиламин, триметиламиноксид, короткоцепочечные жирные кислоты, водород, метан, сероводород.
Конфликт интересов: авторы заявляют об отсутствии конфликта интересов.

Для цитирования: Ивашкин В.Т., Медведев О.С., Полуэктова Е.А., Кудряцева А.В., Бахтогаримов И.Р., Карчевская А.Е. Прямые и косвенные методы изучения микробиоты человека. Российский журнал гастроэнтерологии, гепатологии, колопроктологии. 2022;32(2):19–34. https://doi.org/10.22416/1382-4376-2022-32-2-19-34

Introduction

The number of microorganisms inhabiting the GI tract has been estimated at around 10^{14} [1, 2]. In healthy adults, over 90 % of the gut bacteria belong to the four dominant bacterial phyla such as *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria,* whereas other phyla are present in insignificant amounts [3].

Presently, gut microbiota is regarded as an independent "organ", which regulates multiple metabolic processes in the host organism and has the same importance as other vital organs.

A large part of the gut microbiota functions is performed via metabolic intermediates and end-products. Investigation of the microbial population and its changes associated with various diseases has an evident practical value. This paper provides an overview of the main lines of research into the composition of human gut microbiota.

Genome and transcriptome analysis

Molecular genetic research methods can be classified into several groups.

Polymerase chain reaction (PCR) is the most widely used method of genomic studies. A couple of short complementary stretches of DNA that initiate the PCR reaction (primers) are selected for a target DNA. As a result of the PCR reaction, the target DNA is amplified allowing to detect the PCR product and determine the presence or absence of a specific microorganism in the analyzed sample [4]. A real-time PCR allows to quantify the sample and assess the share of a specific species in the biomass, and monitor the dynamics of its changes during drug

therapy [5]. This method is especially helpful for detecting infectious agents as well as for identifying strains with particular characteristics of interest. For instance, real-time polymerase chain reaction with fluorescent hybridization probes makes it possible to identify and differentiate the DNA of pathogenic *E. coli* strains in clinical samples. However, the method has certain limitations. First, it can be used to detect only known species of microorganisms since it is impossible to generate appropriate primers for an unknown target sequence [6, 7]. Besides, it is technically difficult to identify more than three microorganisms in the course of one PCR reaction.

Targeted sequencing. Essentially, targeted sequencing involves the analysis of one or several target genomic sequences. Since the bulk of human GI microbiota is made up of bacteria, researchers have focused mainly on the 16S rRNA gene codes [8]. Investigations using this method allowed to identify three major enterotypes based on predomination of certain bacterial genera: 1 — *Bacteroides,* 2 — *Prevotella*, 3 — *Firmicutes* [9,10]. Previously, the enterotype was regarded as a stable characteristic of individuals. However, recently obtained data indicate that the enterotype can change over time [11]. Besides, targeted sequencing allows to assess the parameters, which can have importance for clinical practice: namely, "immunity from diseases", diversity of the microbiota composition, the ability of bacteria to synthesize vitamins and produce butyric acid.

Various diseases have certain corresponding patterns of microbiota organization, i.e. consistent patterns of the taxonomic and quantitative composition of microbial communities. While analyzing the results of sample sequencing, the sample profile is compared against the data on several diseases: type II diabetes mellitus, Crohn's disease, ulcerative colitis, obesity, atherosclerosis (including ischemic heart disease). Subsequently, the level of protection against each of the diseases is can be calculated.

Microbiota diversity is critically important: more bacterial species means a higher functional potential of the whole microbiome. In a diverse microbiome, different species are more likely to have complementary functions, able to make up for the species that disappeared as a result of antibiotic therapy or unbalanced diet. If the diversity index is low, this is not the case.

Targeted sequencing allows to assess the capability of gut microbiota to synthesize the following vitamins: vitamin B_2 (riboflavin), vitamin B_1 (thiamine pyrophosphate), vitamin K, vitamin B_9 (folic acid), vitamin B_5 (panthothenic acid), vitamin B_3 (nicotinic acid), vitamin B_6 (pyridoxal 5'-phosphate) и vitamin B_7 (biotin).

In a number of studies, target sequencing was used to analyze the functional activity of gut microbiota. It was shown that the presence of commensal bacteria (e.g. lactobacteria) in the gut microbiota correlates with its capability to synthesize short-chain fatty acids (acetate, butyrate and propionate) [12–16]. Similarly, other researchers developed a system for evaluating the capability of bacteria from human fecal samples to produce trimethylamine (TMA). The system involves investigation of the genes encoding the respective enzyme [17]. Still other studies yielded the results that can be used to reveal a correlation between the composition of microbiota and its capability to synthesize hydrogen, methane and ammonia [18–25].

Among the limitations of this method, one can point out its reduced accuracy due to the presence of similar conservative fragments in the genome of various microorganisms found in the analyzed sample. As a result, while describing a microbial community, it is virtually impossible to use such notions as species, genus, etc. In order to find at least a terminological solution to the problem, an operational definition such as an Operational Taxonomic Unit (OTU) was coined. While describing the results, this term is used as a synonym of a "taxon". There exists a number of bioinformatics resources (PICRUSt, Tax4Fun, Piphillin, FUNGuild) that predicts the functional capabilities of microbial communities based on the taxonomic composition data obtained using targeted sequencing [26, 27].

Errors associated with this method include an insufficient mass of the sample [28], failure to choose a correct variable region or amplicon size [29] as well as insufficient number of PCR cycles preceding sequencing [30].

At the same time, the information about new functions of known members of the gut microbiota is being constantly updated opening up new possibilities for targeted sequencing [31].

Whole genome sequencing. The method entails fragmentation and sequencing of all genomic DNA in a sample. As compared to targeted sequencing, this technique provides more detailed information about the microbial species diversity. At a sufficient sequencing depth, bacteria can be identified within a one-strain accuracy [32, 33]. This method allows to obtain information on the genes encoding proteins and enzymes as well as those involved in the biosynthesis and catabolism of various organic compounds. On the basis of these results, one can derive conclusions about the metabolic potential of microbiota [34, 35]. The main advantage of the method is its usability for examining the microbial community's resistome, i.e. set of antibiotic resistance genes.

Recent years have seen a growing number of intestinal microbiota studies using single-molecule (nanopore) sequencing. This method is absolutely indispensable for the analysis of full genomes of each microorganism in the data pool with subsequent identification of the strains containing a certain functional sequence or resistance factors.

RNA sequencing. It is assumed that a part of bacterial cells in the examined sample are dormant

or present in the form of DNA traces of dead cells. RNA sequencing permits to reveal a metabolically active part of the microbiota. To get an insight into a functionally active part of the microbial community, RNA sequencing data are compared with the data obtained by whole genome analysis [36]. The limitations of the method include high cost, and high risk of RNA degradation associated with improper sample preparation and material storage. These factors affect the reproducibility of results.

Thus, molecular genetic methods of research have become a promising approach to explore the structure of microbial communities and their specific functional characteristics in health and in disease. However, the effectiveness of research using these tools largely depends on the right choice of a technique fit for the goals and objectives of the study.

Metabolome studies

Short-chain fatty acids

Short-chain fatty acids (SCFAs) are the main metabolites produced by the microbiota in the large intestine through the fermentation of indigestible carbohydrates such as cellulose, pectin, xylan or arabinogalactan [37, 38]. The most common SCFAs are acetate, propionate, and butyrate, which differ from each other by the number of carbon atoms (C2, C3 and C4, respectively) [15, 39, 40].

Acetate is the major end product of bacterial breakdown of pectin, xylan and arabinogalactan Bacterial phyla involved in the process of fermentation are: *Verrucomicrobia* (*Akkermansia muciniphila)*, *Bacteroidetes (Bacteroides* spp., *Prevotella* spp.), *Actinobacteria (Bifidobacterium* spp.) и *Firmicutes (Ruminococcus* spp., *Blautia hydrogenotrophica, Clostridium* spp., *Streptococcus* spp.) [41, 42].

Typically, propionate is produced from arabinogalactan through several pathways (succinate, acrylate, propanediol). Microbial phyla responsible for the formation of propionate are the following: *Bacteroidetes (Prevotella ruminicola), Firmicutes (Phascolarctobacterium succinatutens, Ruminococcus flavefaciens, Roseburia inulinivorans, Blautia Anaerostipes rhamnosivorans Lactobacillus reuteri),* and *Proteobacteria (Escherichia coli)* [43, 44].

Butyrate is produced mainly from starch, but acetate and lactate can also serve as substrates for its synthesis [42]. The main butyrate producing-bacteria in the human gut belong to the phylum *Firmicutes (Ruminococcaceae, Lachnospiraceae, Clostridiaceae, Erysipelotrichaceae)* [44].

SCFAs production ratio in the intestine is 3:1:1 for acetate, propionate, and butyrate, respectively [37, 45]. The maximum SCFA concentration was observed in the cecum and proximal colon. Further down the colon, the SCFA concentration gradually declines [42]. SCFAs are partly absorbed by colonocytes and the remaining part enters systemic circulation [46].

SCFA receptors are a subset of G protein-coupled receptors (GPCR) represented by three types of receptors such as GPR43, GPR41 and GPR109A, which differ from one another in their ability to interact with ligands of different length. GPR43 interacts with shorter SCFAs (acetate and propionate); GPR41 does with propionate, butyrate and valerate; GPR109A does mainly with butyrate [46, 47]. Interaction of propionate with GPR41 leads to inhibition of proinglammation agents (such as IL-4, TNF-α and other).

Activation of GPR43 by propionate increases secretion of insulin, glucagon-like peptide-1 (GLP-1), and gut hormone peptide YY (PYY). By activating GPR109A, butyrate inhibits growth of breast tumors facilitating apoptosis in cancer cells [47].

The available research results indicate that a sufficient amount of SCFA produced by gut microbiota is a requisite for maintaining normal functions of the human body. A number of studies examined changes of the microbiota composition and decline in the SCFA production in diabetes mellitus, renal disorders, cardiovascular diseases, cancer, neurodegenerative disease, and obesity [48–64]. However, despite the potential SCFA benefits, a few studies revealed that they have a number of negative effects. Besides, interaction of excess acetate with GPR43 can lead to increased production of interferon gamma (IFN-γ) and IL-17 in the ureter, kidney and draining lymph nodes while naive $CD4^+$ T cells differentiate into Th1 and Th17 cells [65].

A. Tiroshi's study showed that propionate also leads to the development of hyperglycemia in mice by increasing plasma concentrations of glucagon and fatty acid-binding protein 4 (FABP4). A randomized double-blind placebo-controlled human study revealed that consumption of canned foods containing 1 g of calcium propionate (E282) led to insulin-resistance and compensatory hyperinsulinemia [66].

Most studies of SCFA production were conducted using animal models (rodents). However, rodents have certain specific features concerning the composition of GI microbiota, and feeding rhythm (they more often eat at night). These differences make the extrapolation of animal research data to humans a questionable issue [67].

Currently, the number of human studies investigating SCFA production is growing [51, 68, 69]. Many studies focus on measuring SCFA levels in the blood serum and faeces in different population groups (e.g. children, Alzheimer patients) [49, 51]. Still other research involves measuring of SCFA levels in various body fluids and faeces along with the analysis of the microbiota composition [68, 70]. There are also studies investigating the impact of the diet on SCFA production. Thus, an increased content of dietary polysaccharides such as corn and potato

starch as well as inulin led to increases in faecal SCFA concentrations by 32 % and 12 %, respectively ($p < 0.001$). In particular, butyrate was increased by 29 % and acetate, by 21 % [68].

SCFAs are commonly analyzed using gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography (HPLC). Human faeces or, more rarely, plasma and urine are the major samples used for analysis [68, 71]. However, an increased faecal content of a SCFAs may indicate its' impaired absorption rather than overproduction [49]. For this reason, blood plasma may possibly be the best biomaterial for analysis since circulating plasma SCFAs affect metabolic processes [69, 72].

Thus, the issues that confront researchers in the field of SCFA level studies and need further clarification are as follows:

- choice of an optimal biological material for analysis;
- need for a standardized diet before the study;
- selection of an appropriate method for analysis;
- determination of reference ranges corresponding to normal values.

Current research data indicates that acetate, propionate and butyrate are present in a stable ratio of 3:1:1. However, reference range for SCFA concentrations have not been established yet [73].

Trimethylamine (TMA) and trimethylamine oxide (TMAO)

TMA is produced in the colon by microbiota from phosphatidylcholine (PC) and L-carnitine contained in the products of animal origin [74–78].

TMA synthesis from choline involves specific enzymes such as cutC (glycyl radical enzyme GRE choline TMA-lyase) and cutD (activator GRE activase) [79].

These enzymes are encoded by the Cut gene clusters (CutC and CutD being the key ones among them) of intestinal bacteria belonging to the phyla *Firmicutes (Anaerococcus hydrogenalis, Clostridium asparagiforme, Clostridium hathewayi, Clostridium sporogenes), Proteobacteria (Desulfovibrio desulfuricans, Escherichia fergusonii, Proteus penneri, Providencia rettgeri, Edwardsiella Tarda)*, and *Actinobacteria* [77, 80, 81]. The enzymes encoded by these genes break the C-N bond in choline leading to the formation of TMA and acetaldehyde [78, 81].

Carnitine oxidoreductase is the main enzyme responsible for the conversion of L-carnitine into TMA. It is encoded by the pair of genes CntA and CntB, which encode carnitine oxidase and reductase, respectively. The activity of carnitine oxireductase is encoded by gene pair YeaW (Carnitine monooxygenase oxygenase subunit)/YeaX (Carnitine monooxygenase reductase subunit). The main role in these processes is attributed to to the *Proteobacteria* phylum *(Klebsiella pneumoniae, E. coli, Citrobacter, Providencia and Shigella), the class Betaproteobacteria (Achromobacter) as well as the phyla Firmicutes (Sporosarcina) and Actinobacteria* [78].

Another pathway of L-carnitine conversion into TMA includes two steps. First, γ-butyrobetaine (γBB) is formed from L-carnitine by L-carnitine CoA-transferase in the ileum. Then, γBB is converted into TMA by carnitine TMA-lyase in the cecum and colon [82, 83].

Following synthesis in the intestinal lumen, TMA is absorbed from the intestine via passive diffusion across the enterocyte membranes and delivered to the liver where it is converted to trimethylamine N-oxide (TMAO) by hepatic flavin-containing monooxygenase 3 (FMO3) [74]. The FMO3 activity is regulated by bile acids via the bile-acid activated farnesoid X receptor (FXR) [84].

In addition to the pathway whereby TMA is converted into TMAO, the latter can enter the human body directly with TMAO-rich food (seafood, high salt and high fat diet) [85–87]. TMAO can be converted to TMA and the other way around since certain bacteria (e.g. *Escherichia coli*) containing TMAO-reductase [88]. Decreased concentrations can be explained by the presence of the archaea belonging to the order Methanomassiliicoccales and containing the genes encoding methyl-coenzyme M reductase complex capable of reducing the methyl compound of TMA and TMAO [89]. About 95 % TMA is oxidized, and TMA metabolites as well as unchanged TMAO are excreted in urine in the ratio of 3:95 [78].

TMA is capable of binding to G protein-coupled receptors (e.g. trace amine-associated receptor (TAAR5)) and, thus, indirectly involved in the regulation of human behaviour; TMA and TMAO levels can be regulated by sex hormones which allows to regard them as pheromones [78, 84].

TMA is quite rapidly absorbed into the blood stream through the intestinal wall and delivered to the liver where it is converted to TMAO exhibiting a number of biological effects (see Table).

Whether positive or negative TMAO effects prevail is still a matter of debate. Thus, a meta-analysis of 19 studies involving 19256 participants showed that TMAO plasma levels ranged from 3 to 7 umol/l. The optimal TMAO plasma level is <10 umol/l. Higher concentrations (>10–20 umol/l) are regarded as excessive and pathological [96]. The study performed in 2019 and involving people from different countries investigated a possible correlation between an increased death probability and elevated TMAO levels. It was revealed that there is a dose-dependent correlation between the level of mortality risk and TMAO plasma level. The analysis showed that the risk of mortality increased by 7.6 % per each 10 umol/l increment of TMAO [75]. It was also discussed whether high TMAO plasma levels are related to the development of such diseases as psoriasis, atherosclerosis, diabetes mellitus, and osteoporosis. However, not all studies reported a cause-and-effect

relationship between high TMAO levels and these disorders. Therefore, many researchers have been wondering whether it's a cause or a marker of certain diseases [85–87, 94, 95, 97–99]. Furthermore, the results of some studies are conflicting and do not allow to make a definite conclusion that TMAO has negative effects. Thus, the intake of L-carnitine led to increased TMAO level and reduced vessel wall damage in hemodialysis patients [76]. Yin obtained data for patients who had an acute cerebrovascular accident, and transitory ischemic attack. These patients had lower TMAO levels than patients without these disorders though the current concept suggests just the opposite [100].

Many researchers think that it would make sense to modify the diet by reducing consumption of the products containing TMA and TMAO. However, choline leads to decreased neonatal stress and improves placental functioning during pregnancy as well as exerts a positive effect on cognitive function in adults without dementia [77]. Furthermore, the available data indicate that choline in the form of phosphatidylcholine does not increase serum TMAO levels [101]. Besides, choline deficiency can lead to impaired DNA methylation and hepatocellular carcinoma development [102]. Elevated TMAO levels are frequently observed in patients with chronic kidney disease, but it is not quite clear whether TMAO is a marker or a cause of filtering disorders [79, 103]. In Japan consumption of fish containing large amounts of TMAO is associated with increased urinary TMAO observed in the local long-livers. If elevated TMAO levels were linked with increased risk of cardiovascular disease (CVD), a diet rich in fish should have been associated with high CVD risk. However, it is not in this case since there are lots of evidence that high fish intake is beneficial to the cardiovascular system [76, 78].

TMAO level can be measured in the urine, blood and faeces. Liquid chromatography and high-resolution mass spectrometry are the most common tools used for this purpose. However, preparation of an appropriate substrate for HRMS is an extremely complex task [104]. Besides HRMS, procedures applied for measurement of TMA and TMAO in the plasma and urine include proton nuclear magnetic resonance spectroscopy, gas chromatography, ionization mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry [79].

However, it is still unclear whether elevated TMAO concentrations associated with certain disorders result from cellular adaptation to stress and, hence, can serve as a marker and prognostic indicator for a particular disease, or it can be regarded as one of the causes of a fairly large number of diseases [105, 106].

Intestinal gas production studies: hydrogen, methane and hydrogen sulfide

Intestinal gases reflecting the composition and functional potential of gut microbiota such as hydrogen (H_2), methane (CH_4) and hydrogen sulphide (H_2S) are partly absorbed from the intestine into the blood and then excreted with exhaled air which makes it possible to measure their concentrations and obtain indirect data on the gut microbiota composition.

Hydrogen

A molecule of hydrogen is composed of two hydrogen atoms. It is non-polar and electrically neutral. Hydrogen molecules easily penetrate inside intracellular structures (mitochondria, nucleus, etc.) and pass through the blood-brain barrier (BBB).

Hydrogen accounts for about 19 to 20 % of all gases produced in the colon [107] while its production rate is 100-fold higher than that in the small intestine [108].

According to modern views, the cells of mammals including humans are incapable of producing molecular hydrogen [108, 109]. It is assumed that intesti-

Table. Biological Effects of TMAO

Positive effects	Negative effects
Maintenance of cell volume (osmolyte) [76, 78, 90]	Reduction of bile acid synthesis, diminished excretion of cholesterol from the body [77, 79, 90, 91]
Reduction of endoplasmic reticulum stress [77, 85]	High concentrations of TMAO enhance platelet aggregation [83, 92, 93]
Provide protection from prion diseases [77, 85]	Proinflammatory effect due to increased expression of proinflammatory cytokines and proinflammatory pathway activation [76, 90]
Stabilization of protein structure (chaperon function) [77]	Endothelial dysfunction [90, 94]
Decreased activity of the MAPK/ERK and NF-kB pathways [90]	Increased virulence of *Helicobacter pylori* due to enhanced expression of the CagA virulence genes [86]
	Increased permeability of the blood brain barrier (BBB) [92]
	Neurodegeneration [95]

nal hydrogen is produced primarily by such bacteria as *Ruminococcus spp., Roseburia spp., Clostridium spp.* belonging to the phylum *Firmicutes; Bacteroides spp* belonging to the phylum *Bacteroidetes* [110, 111]. Furthermore, over 200 pathogenic organisms have the ability to synthesize hydrogen [112]. Inclusion of non-digestible carbohydrates in the diet leads to increased hydrogen production by the gut microbiota [108, 110, 111, 113]. However, this increase is not accompanied with a growing share of hydrogen-producing microorganisms while a high positive correlation was found between the hydrogen production and the amount of *Bifidobacteriales* (phylum *Actinobacteria*) not involved in the hydrogen synthesis [114].

The results of rodent studies revealed a significant difference between the amount of hydrogen produced by gut microbiota of experimental rats from different colonies on the background of the equal intake of dietary fiber. Thus, portal blood hydrogen concentration in the first group of rats amounted to 1.54 umol/l versus 17.4 umol/l in the second group. Per oral transplantation of rat colonic microbiota with high H_2 production to the low H_2-generating animals of the first group led to an increase of the H_2 concentration in the portal vein from 3.07 to 9.95 umol/l as well as a growing number of bacteria belonging to the genera *Bifidobacterium* (phylum *Actinobacteria*), *Allobaculum* (phylum *Firmicutes*) and *Parabacteroides* (phylum *Bacteroidetes*). At the same time, there was a decrease in the levels of *Bacteroides* (phylum *Bacteroidetes*), *Ruminococcus* (phylum *Firmicutes*) and *Escherichia* (phylum *Proteobacteria*) [114]. The *Bifidobacterium* cannot produce hydrogen since they lack hydrogenase. The authors assume that *Bifidobacterium* generate acetate and lactate while metabolizing dietary fiber. Lactate is utilized by other bacteria producing acetate and butyrate. Therefore, while *Bifidobacterium* themselves do not produce H_2, a 50 % increase in their number following the transplantation can facilitate higher H_2 generation by other bacteria. Similar results were obtained by Japanese researchers in the studies involving human subjects [115]. The mechanisms and exact type of relationship between an increased H_2 generation under the impact of dietary fiber and growth of certain bacterial species still remain unclear and require further research.

Research findings substantiating the antioxidant activity of hydrogen in biological models were published in 2007 [116].

Subsequent studies showed that hydrogen protects the brain during cerebral ischemia/reperfusion and stroke [117]; antiatherosclerotic H_2 effect was also demonstrated in the experiments on mice [118]; cardioprotective H_2 effect was seen in a myocardial ischemia/reperfusion model [119]. Still other researchers observed anti-stress effect of high hydrogen concentrations in the experiments on lab mice [120] as well as protective hydrogen effect in pulmonary hypertension models [121, 122]. Furthermore, the obtained evidence shows that hydrogen has an impact on signalling pathways whereby information is transmitted across the cell membrane as well as exerts cytoprotection, and decreases the synthesis of proinflammatory cytokines and apoptosis [123–125]. Subsequent clinical trials undertaken to test the antioxidant properties of H_2 largely confirmed the results of previous experimental studies on animals. Thus, researchers corroborated cardioprotective and neuroprotective effects of H_2 [126–130] as well as a positive hydrogen effect on endothelial dysfunction [131, 132]. H_2-enriched dialysis solution improved the prognosis of both hemodialysis and peritoneal dialysis patients by decreasing the development of fibrosis [133–135].

On the one hand, the discovery of positive effects of molecular hydrogen led to an increase in the number of studies investigating exogenous hydrogen effects and, on the other, revived interest to the analysis of effects of endogenous hydrogen produced by gut microbiota.

There is a linear correlation between the hydrogen intestinal production rate and its concentration in exhaled breath: from 21 % to 65 % of hydrogen produced in the gut is absorbed into the blood and excreted through lungs which provides a sufficient basis for using a hydrogen breath test to assess the level of gas production by gut microbiota [108, 136, 137].

Hydrogen concentration in exhaled air is measured to determine GI transit time, diagnose small bowel bacterial overgrowth syndrome, lactase insufficiency, and carbohydrate intolerance (fructose, galactose, sorbitol) [138–141]. However, a large data spread is observed in most studies. This variability does not allow to draw conclusions about reference ranges for breath hydrogen concentrations in healthy subjects [142]. A number of current studies are attempting to provide a sufficient basis for the development of clinical guidelines with a view to standardize hydrogen breath testing and interpretation of test results in clinical conditions [143, 144].

Thus, further studies investigating the relationships between the gut microbiota composition and hydrogen concentration in the expired air as well as development and standardization of methods for measuring breath hydrogen levels in healthy subjects and patients with different diseases appear to be rather a promising line of research.

For example, researchers from Moscow State University (Faculty of Chemistry) jointly with their colleagues from IRZ-Locomotiv LLC (affiliated company of Izhevsky Radiozavod JSC) are currently working on the research project entitled “Development of a method for measuring hydrogen concentration in exhaled breath using semiconducting metal oxide-based sensors”. This method will be used to design a hydrogen analyzer in two versions: a portable analyzer for home use and a professional version for healthcare professionals.

Methane

Methanobrevibacter smithii and *Methanosphaera stadtmanae* are the main methane producers in human colon. Their number increases all along the colon until reaching a maximal value in the rectum [145]. *Methanobrevibacter smithii* use hydrogen to reduce CO_2 to methane whereas *Methanosphaera stadtmanae* use hydrogen for reduction of methanol to methane [146, 147]. Four hydrogen molecules and one CO_2 molecule are required to synthesize one molecule of methane.

The number of methanogens in the gut microbiota changes with age. Typically, the gut microbiota of children has a low content of methanogens children's microbiota (10^3–10^6 CFU/1 g of faeces) [147–149]. The share methanogens grows with age reaching 60 to 80 % of the total GIT microbial population in the age group 80–90 [150–153].

Differences in breath methane concentrations can be associated with the diet, geography and a number of other factors. For example, patients who had undergone appendectomy had decreased levels of exhaled methane. Presumably, this finding is explained by the fact that the appendix may serve as a reservoir for methanogens [154]. Increased exhaled methane concentrations exceeding the background level were observed only in 15 % of Japan's population while *Methanobrevibacter smithii* were found just in 8 % of the Japanese [155]. A comparative study of exhaled methane levels in Native Africans versus American Africans showed that the latter have much lower exhaled methane levels and a substantially smaller proportion of methanogens in the gut microbiota [156, 157]. These findings suggest that dietary patters may have an impact on the composition of gut microbiota.

On the whole, studies revealed significant differences in breath methane levels and it was deemed expedient to single out two distinct groups of subjects, i.e. with high and low breath methane levels, respectively. The first group is made up by the individuals whose breath methane concentration is 4 ppm or higher and exceeds the atmospheric methane concentration by 1 ppm; the second group included subjects with the breath methane levels below 3 ppm [151]. High breath methane concentration indicates the presence of methanogens enumerated at the order of 100 CFU per 1 g of faeces which corresponds to a 0.03 to 0.3 % of the whole gut microbiota [158, 159]. High concentration of methanogens in the colonic microbiota and elevated concentration of hydrogen in exhaled air are associated with a higher body mass index and visceral fat percentage [160, 161]. The latter is a more significant risk factor for metabolic disorders and heart disease than body mass index or waist circumference [162, 163]. Furthermore, increased breath methane concentration has a direct positive correlation with the severity of constipation, and negative correlation with diarrhoea severity in patients with irritable bowel syndrome [143, 164]. However, according to the data obtained by Singh et al, correlation with the severity of constipation is observed only at high methane baseline concentrations of 10, 20 ppm or higher ($p < 0.001$) [165].

Methane concentration in exhaled air is normally measured using a methane breath test. However, test administration is complicated by the lack of standardized procedures as well as the fact that methanogenic bacteria are present not only in the colonic microbial population but also in the oral microbiota [166, 167].

Thus, currently available data do not allow to make unambiguous conclusions about the feasibility of using methane concentration measurement in exhaled air for diagnostic purposes. Further studies are required to investigate relationships linking the concentration of methanogenic bacteria in the gut microbiota to the factors determining methane concentration in exhaled air as well as to the altered methane production levels seen in certain diseases.

Hydrogen sulphide (H_2S)

Hydrogen sulphide (H_2S) formed through bacterial breakdown of proteins and other sulphur-containing substances can also serve as a biomarker of the gut microbiota composition. However, in contrast to hydrogen and methane, hydrogen sulphide is synthesized not only by the microbiota, but also somatic cells of the host organism. For this reason, it is a less specific marker of the GI microbiome composition.

Sulfur present in the human body is obtained from dietary animal and plant-based proteins as well as from drinking water containing inorganic sulfur compounds [168]. Dietary proteins and peptides, that remain undigested in the small intestine by proteases and peptidases, reach the colon and constitute a source of sulfur-containing amino acids [169]. Besides, there are endogenous sulfur-containing glycoproteins with a molecular mass of about 2.5 MDa, which serve as a main component of gel-forming MUC2 mucin [170].

Sulfate-reducing bacteria (SRB) belong to the phyla *Protobacteria* (*Escherichia, Desulfovibrio, Klebsiella, Salmonella, Enterobacter*), *Fusobacteria* (*Fusobacterium*), *Firmicutes* (*Clostridium, Streptococcus*) [171]; they have the ability to synthesize H_2S from methionine, cysteine and taurine [172]. These bacteria are found in the human gut microbiota in 15 % of children and 50 to 60 % of adults [159, 173]. Similarly to methanogenic bacteria, sulfate-reducing bacteria use hydrogen to synthesize the end product. Synthesis of one H_2S molecule involves five hydrogen molecules [174]. SCFAs represent the main source of carbon for most sulfate-reducing bacteria: 14.1 %, 9 %, and 9 % is provided by acetate, propionate, and butyrate, respectively; and lactate serves as a major substrate for 63 % of SRB.

In human tissues endogenous H_2S synthesis involves three main enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) [175, 176]. Two vitamin B_6 — dependent enzymes, CBS and CSE, are localized in the cytosol whereas 3-MST resides both in the cytosol and mitochondria. All enzymes involved in H_2S synthesis were found in most human organs and tissues including vascular and pulmonary endothelium, smooth muscle cells, adipose tissue, brain, heart and lungs [177, 178].

Flannigan et al. studied the relative proportion between the amount of H_2S synthesized by sulfate-reducing bacteria residing in the gut and by somatic cells of the host organism. It was shown that the amount of H_2S in the faecal material of germ-free mice was two-fold lower than in the animals with normal gut microbiota. The diet devoid of vitamin B_6, a cofactor for the two key enzymes involved in tissue H_2S synthesis, led to a 50 %-decrease in the amount of the synthesized H_2S. The authors came to the conclusion that somatic cells and sulfate-reducing bacteria produce equal amounts of H_2S [179].

Further studies examined plasma free H_2S levels in various organs as well as in germ-free mice versus conventional mice. It was demonstrated that plasma H_2S concentration of germ-free mice was 2.5-fold lower as compared with regular mice [180]. The largest differences in free H_2S levels between the two groups of mice were seen in the GI tissues while the H_2S levels in the lung tissue were the same. Since blood H_2S concentration is a major factor determining its diffusion into the alveolar air, it can be assumed that the values obtained by measuring the H_2S level in exhaled air would largely reflect the activity of sulfate-reducing bacteria.

Recently, researches into H_2S involvement in the regulation of inflammation and oxidative stress as well as infectious and oncological diseases have been attracting much interest [177, 178, 181]. Hydrogen sulphide produced by aerobic and anaerobic bacteria in the oral cavity can be responsible for periodontitis and halitosis [182, 183].

There are multiple methods for measuring hydrogen sulphide concentrations in exhaled air described in the literature: carbon nanotubes, fluorescent probes, high-performance liquid chromatograpy, etc.

The fact that H_2S is produced both by the human gut microbiota and somatic cells makes it a less specific biomarker of the gut microbial enzyme activity. Apparently, due to its low concentration in the intestinal lumen, H_2S plays a very small role in utilizing hydrogen as compared with methane. Nonetheless, H_2S studies examining it both as a biomarker and a substance involved in the regulation of multiple metabolic pathways, oxidative stress, and mitochondrial functions appear to be very promising lines of future research in this area.

Conclusions

Modern studies of the gut microbiota have clearly demonstrated and made it increasingly apparent that the role of a microbial consortium predominates over specific functions of individual bacterial species.

Despite the fact that currently molecular genetic methods are mostly employed in fundamental research and lack a unified protocol for data analysis, there is a tendency to transfer these techniques in clinical practice.

Measurement of SCFA concentrations (acetate, propionate and butyrate) in plasma provides the data, which can serve as an indirect biomarker of the colonic microbiota composition. However, currently available evidence is insufficient to relate the obtained values (SCFA levels and ratio) to a particular disease with a high degree of certainty. TMAO levels in the blood plasma and urine can also reflect the presence of specific bacterial clusters containing genes Cut, CntA/CntB and YeaW/YeaX. Therefore, further studies are required to reveal possible correlations between certain disorders and such parameters as the composition of gut microbiota, dietary patterns and TMAO concentration.

Gas biomarkers, i.e. hydrogen, methane and hydrogen sulphide, have been studied in more detail and are better understood as compared to other biomarkers of the gut microbiome composition and functionality. The main advantage of gas biomarkers is that they can be measured multiple times using non-invasive techniques. These measurements provide information on the relative proportion of hydrogenic (i.e. hydrogen producing) and hydrogenotrophic (i.e. methanogenic and sulfate-reducing) microorganisms. In its turn, this opens up the possibility of developing new approaches to correction of individual microbiota components.

Integration of the data obtained by gut microbiota studies would allow a more comprehensive analysis of microbial community function and its interaction with the human organism. This approach appears to be very promising and may increase our knowledge and understanding of the pathogenesis of various diseases as well open up new opportunities for prevention and treatment of these disorders.

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Поступила: 16.12.2021 Поступила после исправления: 15.02.2022 Принята: 25.02.2022
Опубликована: 15.05.2022
Submitted: 16.12.2021 Revision received: 15.02.2022 Accepted: 25.02.2022 Published: 15.05.2022

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