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COMPREHENSIVE PROFILING OF ORAL MUCOSAL MICROBIOTA BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY OF MICROBIAL MARKERS

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КОМПЛЕКСНАЯ ХАРАКТЕРИСТИКА МИКРОБИОЦЕНОЗА СЛИЗИСТОЙ ОБОЛОЧКИ ПОЛОСТИ РТА С ПРИМЕНЕНИЕМ ГАЗОВОЙ ХРОМАТОГРАФИИ/МАСС-СПЕКТРОМЕТРИИ МИКРОБНЫХ МАРКЕРОВ

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The study presents a comprehensive analysis of the oral mucosal microbiota in apparently healthy adults, which is performed with gas chromatography/mass spectrometry (GC/MS) of microbial markers. The examined population (n=69, aged 21–59) featured a significant taxonomic and quantitative diversity of the oral microbiota along with lack of clinical signs of pathology. The dominant phyla of the microbiota were *Bacillota* (*Firmicutes*) and *Actinomycetota* (*Actinobacteria*). Among the resident microorganisms, *Clostridium spp.*, *Rhodococcus spp.*, *Nocardia asteroides*, *Aspergillus spp.*, *Micromycetes spp.*, and *Lactobacillus spp*. were frequently detected. A distinctive feature of the identified spectrum is the predominance of transient species such as *Streptococcus spp.* and *S. mutans*, which calls for a revision of traditional views on their role in shaping the oral microbiota in healthy individuals. The obtained reference values for the content of key microbial markers may serve as a basis for subsequent personalized diagnostics and monitoring of the effectiveness of preventive and therapeutic interventions in dental practice. The results presented demonstrate a high degree of individual variability in the oral microbiota, underscoring the need for an individualized approach to the assessment of microbiological data in clinical settings.

Keywords: personalized medicine, oral mucosal microbiota, gas chromatography/mass spectrometry, microbial markers, microbiocenosis

Представлен комплексный анализ микробиоценоза слизистой оболочки полости рта у практически здоровых взрослых с применением газовой хроматографии/масс-спектрометрии микробных маркеров. В обследованной популяции (n=69, возраст 21–59 лет) установлено значительное видовое и количественное разнообразие микробиоты при отсутствии клинических проявлений патологии. Доминирующими филумами биоценоза были *Bacillota* (*Firmicutes*) и *Actinomycetota* (*Actinobacteria*). Среди резидентных микроорганизмов часто выявлялись *Clostridium spp., Rhodococcus spp.*, *Nocardia asteroides, Aspergillus spp.*, *Micromycetes spp.*, *Lactobacillus spp.* Характерной особенностью выявленного спектра является преобладание транзиторных видов *Streptococcus spp.* и *S. mutans*, что требует пересмотра традиционных представлений об их роли в формировании микробиоценоза у здоровых лиц. Полученные референс-значения содержания основных микробных маркеров могут служить основой для последующей персонализированной диагностики и мониторинга эффективности лечебно-профилактических мероприятий в стоматологической практике. Результаты свидетельствуют о высокой межиндивидуальной вариабельности микробиоты полости рта, что подчёркивает необходимость индивидуального подхода при оценке микробиологических данных в клинике.

Ключевые слова: персонализированная медицина, слизистая оболочка полости рта, газовая хроматография/масс-спектрометрия, микробные маркеры, микробиоценоз

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GC/MS – gas chromatography/mass spectrometry

The human oral cavity represents a complex ecosystem offering a favorable environment for numerous species of bacteria, archaea, viruses, fungi, and unicellular eukaryotes [1]. The specific features of the oral cavity microbiocenosis are determined by the relatively stable microbial populations to be found on the mucous membrane of the cheeks, gums, tongue, in the gingival pockets, as well as on the teeth. Multi-species communities differ in composition and spatial arrangement, and as they interact with each other and with the human host, they form a remarkably complex biofilm [2–4].

Since the discovery of the first microorganisms in 1683 by Antonie van Leeuwenhoek, cultivation has remained the leading method employed to study the human microbiota. The method in question has long been considered expensive and time-consuming, the result of that being newer molecular approaches (metagenomics and polymerase chain reaction) getting more and more widely used. At the same time, the obtained outcomes have been difficult to reproduce [5].

The methods described in the respective literature can facilitate a comprehensive study of the microbial composition of the complex ecosystem; however, studies of the oral mucosa microbiota remain scarce [6]. Currently, there is a lack of comprehensive understanding of the qualitative and quantitative diversity of microbiocenosis that can be applied in practical dentistry.

The year 1993 saw the development of an express method for identifying microorganisms in biological samples using gas chromatography/mass spectrometry (GC/MS) [7]. In contrast, the method relies on the fact that microbial cells produce short-chain fatty acids, aldehydes, and sterols during metabolism, and these compounds are specific to a particular microorganism. The method is used to examine the species composition of microorganisms inhabiting various anatomical areas (intestines, oropharynx) and human biosubstrates (blood, urine) [8–11].

This study aimed to investigate the microbiocenosis of the oral mucosa using GC/MS to analyze microbial markers, with subsequent reconstruction of the microbial community.

Material and Methods. The study involved 69 virtually healthy patients aged 21 to 59 (median age, 40.9±1.6 years) who regularly visited dentists for preventive purposes. 25 (36.2 %) of the patients were men, with the other 44 (63.8 %) being women. Virtually healthy patients were those who, at the time of the examination, expressed no complaints and had no objective signs of visible pathological issues affecting the mucous membranes; the patients had their oral cavities sanitized. The hygienic status was assessed as satisfactory (subject to the Green – Vermillion index).

The material for studying the qualitative and quantitative composition of the microflora was selected based on the patients' informed consent. Samples were taken from the mucous membrane of the retromolar area of the oral cavity, and a sterile viscose probe was used. The probes were then placed in sterile Eppendorf tubes to be delivered to the laboratory on the same day.

Markers of fatty acids, aldehydes, and sterols were detected by GC/MS using the 7820N-5975 Agilent Technologies (USA) system equipment; the microbial community reconstruction was done with special MSD Productivity ChemStation software and the NIST 2014 mass spectrum library at a microbial chromatography laboratory (MedBasis, LLC; St. Petersburg, Russia). The area of the marker chromatographic peak was proportional to its concentration, which, therefore, means it was proportional to the concentration of the respective microorganism, which was identified as the number of cells contained in one gram of biomass, subject to the formula presented in [8].

The microbiocenosis identification of a biotope included the calculation of the proportion of microorganisms, both permanently present (resident microflora) and transient ones, whose composition may change while influenced by endogenous (heredity, pregnancy) and exogenous (diet, alcohol or tobacco use, socio-economic status, use of antimicrobial medications) factors [12, 13]. Microorganisms found in more than 50 % of the samples were classified as resident, whereas those found in less than 50 % were classified as transient.

The statistical data processing was carried out using the MS Excel 2019 software (Microsoft, USA), a method involving the preliminary «purification» of samples to achieve a normal distribution with a normalized standard deviation. This was followed by the calculation of a 95 % confidence interval, resulting in the average sample values (M) and their acceptable deviation intervals (m) for each microorganism, which are shown in the text as M±m. The differences between the studied signs were considered statistically significant at $p<0.05$.

Results and Discussion. The GC/MS study of samples from the mucous membrane of the retromolar area helped identify fatty acids, aldehydes, and sterols, all of which correspond to the microbial markers of 48 out of 58 species, genera, or groups of microorganisms, which is possible when employing the said method. The reconstruction of the microbial community members revealed representatives of 18 (37.5 %) species or genera of the *Bacillota* (*Firmicutes*) phylum, 13 (27.1 %) of *Actinomycetota* (*Actinobacteria*), 5 (10.9 %) of *Bacteroidota* (*Bacteroidetes*), with another 6 (12.5 %) species of microorganisms classified as *Fusobacteriota* (*Fusobacteria*) and *Pseudomonadota* (*Proteobacteria*). The groups of micromycetes – 4 (8.3 %) and viruses – 2 (4.2 %) were less diverse.

The bacteria isolated from the surface of the oral mucosa were predominantly anaerobic and optionally anaerobic (n=31; 73.8 %), while the aerobic ones (n=11) accounted for 26.2 %. Similar data on the predominance of anaerobic bacteria in the material obtained through cultivation can be found in [14]. The Human Oral Microbiome Database (HOMD), based on bacteriological and molecular genetic studies of the human oral microflora, also offers data concerning the prevalence of the *Bacillota* (*Firmicutes*) bacteria type, including the *Clostridium* genus [15].

The average microbial content in the sample was 30585×10^5 cells/g, with the *Bacillota* (*Firmicutes*) phylum bacteria ($n=17283\times10^5$ cells/g; 56.5 %) and micromycetes ($n=9106\times10^5$ cells/g; 29.8 %) prevailing, whereas the content of bacteria belonging to the *Actinomycetota* (*Actinobacteria*) (10.6 %), *Bacteroidota* (*Bacteroidetes*) (0.3 %) and other (0.3 %) phyla, as well as viruses (2.5 %) was lower (Fig. 1).

As the study here showed, the quantitative content of micromycetes was significant. When measured, for instance, using the sitosterol marker, the content was $(13524\pm2840)\times10^5$ cells/g, and with the campesterol marker, it was $(2121\pm2188)\times10^5$ cells/g. The number of *Aspergillus* genus micromycetes cells, however, reached 2335 (2017)=10^5 cells/g. As far as anaerobic bacteria are concerned, the most significant proportion was accounted for by species such as *C. perfringens* $(4.057\pm3.616)\times10^5$ cells/g, *C. tetani* $(3.196\pm2.985)\times10^5$ cells/g, and the *Lactobacillus* genus $(2.980\pm2.662)\times10^5$ cells/g.

The variability of individual indicators implied a high level of standard deviation from the average values. It is interesting to note that, from a clinical standpoint, the most significant microorganisms responsible for caries development (the *Streptococcus* genus) were observed in much lower amounts – $(9\pm6)\times10^5$ cells/g. In contrast, the levels of the *S. mutans* species were $(825\pm340)\times10^5$ cells/g (Table).

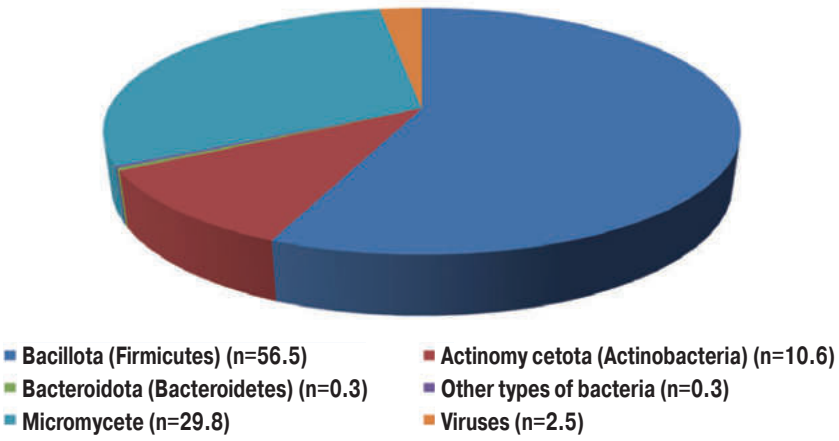

Fig. 1. Quantitative content of microorganism groups in samples obtained from the mucous membrane of the retromolar area (n=30585 x 10^5 cells/g)

All patients who underwent examination were found to have microbial markers typical of *C. tetani, Rhodococcus spp., Nocardia asteroides*, and *Aspergillus spp.* In addition, more than 90 % of the examined individuals had *Actinomyces viscosus, Micromycetes spp., Clostridium perfringens*, and *Lactobacillus spp*. detected in them. Calculations showed that 22 species (genera) of microorganisms were classified as resident, and 26 as transient. Ten species of anaerobic bacteria were found among resident microorganisms (frequency rate above 50 %), with the genus of *Clostridium* (*C. tetani, C. perfringens, C.* (*Blautia*) *coccoides, C. ramosum, C. propionicum, C. difficile*) prevailing among them.

Earlier, when analyzing the distribution of *Streptococcus spp.,* it was shown that they reside in different areas of the human oral cavity: *S. Mitis* is found on the mucous membrane of the cheeks, *S. oralis is* in plaque, and *S. infantis is* on the back of the tongue [16]. In our study, the *Streptococcus* genus microorganisms were classified as transient based on their discharge rate, which ranged from 17 % for *Streptococcus spp.* to 45 % for *Streptococcus mutans*.

The frequent occurrence of the microorganism did not always correlate with its high content. Several microorganisms isolated from the overwhelming majority of the individuals proved the contrary to be true – they featured insignificant quantities. The vast majority of the patients, for instance, had *C. coccoides* (80 %), *A. viscosus* (94 %), and *N. asteroids* (99 %), yet their number was tenfold as low: $(138\pm122)\times10^5$ cells/g, $(841\pm695)\times10^5$ cells/g and $(723\pm515)\times10^5$ cells/g, respectively (Fig. 2).

An inevitable part of the oral cavity ecosystem is its normal microflora, which is a well-arranged interacting community of microorganisms functioning as a primary target for any factors, while offering colonization resistance [17]. The oral microbiome is the second-largest and most diverse human microbiota, surpassed only by the microbiota of the intestines [18]. Constant contact with the outer environment and proper humidity create favorable conditions for the adhesion, colonization, and reproduction of aerobic and anaerobic microorganisms, which mainly belong to streptococci (*S. salivarius, S. sanguis, S. mutans*), lactobacilli (*L. casei, L. acidophilus, L. salivarius*), gram-negative anaerobic and microaerophilic bacteria of the *Bacteroidaceae* family (*B. melaninogenicus and B. gingivalis*), *Fusobacterium, and Propionibacteriaceae* [17].

Table

Detection rate and levels of microorganisms in samples obtained from the mucous membrane of the retromolar area (n=69)

Group		№	Microorganisms	Detection rate (%)	Level, $\times 10^5$ cells/g
Bacteria	*Bacillota (Firmicutes)* phylum	1	*Bacillus cereus*	14	35±21
		2	*Bacillus megaterium*	16	10±6
		3	*Clostridium (Blautia) coccoides*	80	138±122
		4	*Clostridium difficile*	55	597±515
		5	*Clostridium perfringens*	93	4057±3616
		6	*Clostridium propionicum*	61	165±153
		7	*Clostridium ramosum*	83	1463±1312
		8	*Clostridium tetani*	100	3196±2985
		9	*Enterococcus spp.*	1	0±0
		10	*Eubacterium spp.*	67	2234±1597
		11	*Lactobacillus spp.*	90	2980±2662
		12	*Peptostreptococcus anaerobius* 17642	0	0±0
		13	*Peptostreptococcus anaerobius* 18623	41	34±15
		14	*Ruminococcus spp.*	70	466±323
		15	*Staphylococcus aureus*	68	973±830
		16	*Staphylococcus epidermidis*	54	101±85
		17	*Streptococcus mutans*	45	825±340
		18	*Streptococcus pneumonia*	7	0±0
		19	*Streptococcus spp.*	17	9±6
	Actinomycetota (Actinobacteria) phylum	20	*Actinomyces spp.*	91	105±75
		21	*Actinomyces viscosus*	94	841±695
		22	*Bifidobacterium spp.*	46	325±109
		23	*Corynebacterium spp.*	42	77±29
		24	*Eggerthella lenta*	65	357±264
		25	*Nocardia asteroides*	99	723±515
		26	*Nocardia spp.*	9	34±23
		27	*Propionibacterium acnes*	7	0±0
		28	*Propionibacterium freudenreichii*	29	260±118
		29	*Propionibacterium jensenii*	0	0±0
		30	*Propionibacterium spp.*	29	7±4
		31	*Pseudonocardia spp.*	81	124±107
		32	*Rhodococcus spp.*	100	376±271
		33	*Streptomyces farmamarensis*	0	0±0
		34	*Streptomyces spp.*	32	6±3
		35	*Mycobacterium spp.*	0	0±0
	Bacteroidota (Bacteroidetes) phylum	36	*Bacteroides fragilis*	17	8±4
		37	*Bacteroides hypermegas*	7	1±1
		38	*Flavobacterium spp.*	0	0±0
		39	*Porphyromonas spp.*	3	0±0
		40	*Prevotella ruminicola*	14	27±16
		41	*Prevotella spp.*	14	69±37
	Other phyla (*Fusobacteriota, Pseudomonadota*)	42	*Alcaligenes spp.*	30	65±31
		43	*Campylobacter mucosalis*	0	0±0
		44	*Chlamidia trachomatis*	0	0±0
		45	*Enterobacterales (E. coli, etc.)*	1	0±0
		46	*Fusobacterium/Haemophylus*	42	9±4
		47	*Helicobacter pylori*	1	0±0
		48	*Kingella spp.*	0	0±0
		49	*Moraxella spp.*	7	7±5
		50	*Pseudomonas aeruginosa*	10	5±4
		51	*Stenotrophomonas maltophilia*	0	0±0
Micromycetes		52	*Aspergillus spp.*	99	2335±2017
		53	*Candida spp.*	79	1126±1152
		54	*Micromycetes spp.* (campesterol)	93	2121±2188
		55	*Micromycetes spp.* (sitosterol)	91	3524±2840
Viruses		56	*Human alphaherpesvirus 1, 2 (HHV-1, 2)*	74	704±620
		57	*Human betaherpesvirus 5 (HHV-5)*	0	0±0
		58	*Human gammaherpesvirus 4 (HHV-4)*	10	75±54

The oral cavity has been proven to have around 774 bacterial species, mainly representing several dozen genera of seven phyla: *Actinomycetota* (formerly *Actinobacteria*), *Bacteroidota* (*Bacteroidetes*), *Bacillota* (*Firmicutes*), *Fusobacteriota* (*Fusobacteria*), *Pseudomonadota* (*Proteobacteria*), *Saccharibacteria* (*TM7*), *and Spirochaetota* (*Spirochaetes*) [19]. Approximately 54 % have been described and have a specific name, while 14 % are cultivated but have not been described, and another 32 % are neither cultivated nor described [20]. The availability of nutrients, the host's immunity, the oxygen content, and the temperature determine the localization of bacteria as well as the development of complex bacterial communities [21].

The oral mucosa of a healthy person is dominated by *Streptococcus, Granulicatella, Neisseria, Haemophilus, Corynebacterium, Rothia, Actinomyces, Prevotella, Capnocytophaga, Porphyromonas*, and *Fusobacterium* [15]. However, once the status changes, this microbial balance is disrupted, thereby increasing the levels of certain types of bacteria, which, in turn, trigger the pathogenesis of various diseases, including dental caries, periodontitis, and endodontic infections [10, 18]. Recent molecular studies have also demonstrated that the oral microbiota is diverse and complex, playing a crucial role in the pathogenesis of dental and systemic diseases [11, 22, 23].

The traditional method of identifying etiologically meaningful bacteria that colonize the human oral mucosa is bacteriological diagnostics, which involves bacterioscopy of the material, a bacteriological study aimed at isolating pure cultures of pathogens, and, finally, their identification by the type of substrate fermentation [14]. High efficiency has been observed in the case of a combination of the culture method with that of the polymerase chain reaction [22].

Metagenomics has made a significant contribution to the expansion of knowledge about the uncultivated portion of the human microbiota, which does not match any known microbe [6, 15]. Recent years have witnessed a paradigm shift in the study of the human microbiota, with improved bacteriological approaches making a comeback.

The method we employed involves the identification of microorganisms through the detection of specific fatty acids, aldehydes, and sterols in biological samples [7, 24]. It is similar to genetic analysis (PCR, 16S-rRNA nucleotide sequencing, etc.), as the composition of fatty acids is encoded

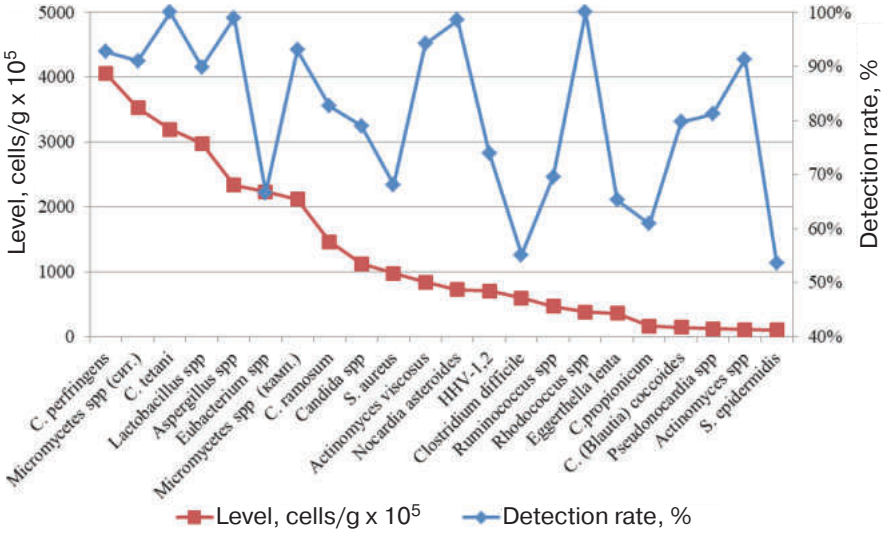

Fig. 2. Comparative description of the isolation frequency and quantitative content of resident microorganisms in samples obtained from the mucous membrane of the retromolar area

in DNA and replicated through the replication of a section of the genome with tRNA, followed by the further synthesis of fatty acids in mitochondria using mRNA. The types of bacteria that account for the majority of the oral microbiome tend to persist in different individuals. Differences in the relative abundance of taxa, as well as variations at the strain level, the presence of rare strains, and species, account for most of the diversity at the gene level, while also enabling the identification of individual features [25].

A possible approach to solving the issue in question is the GC/MS method, which, when employed to study samples from the oral mucosa, has shown that individual indicators of microbiocenosis of this biotope include aerobic and anaerobic bacteria, viruses, and micromycetes in various qualitative and quantitative combinations.

Conclusion. Currently, research projects are being conducted that aim to detect and identify oral microorganisms; however, methods used for assessing microbiocenosis, which are available in practical dentistry, have not been described. Bacteriological and molecular genetic methods are widely used. In contrast, the GC/MS method has established standards for identifying microbial communities in various human anatomical areas based on the qualitative and quantitative content of specific chemical markers indicative of microbial presence. During this time, there is a need to develop local software and expand databases of human microorganism mass spectra.

This study, which employed the GC/MS method, focused on examining the microbial landscape of the oral mucosa in the retromolar region of individuals with virtually no dental pathology. Additionally, the average qualitative and quantitative specific features of microbiocenosis were identified. The outcomes obtained through this study can serve as reference values in dental practice for individual comprehensive evaluation and study of changes in the composition and ratio of microorganisms affected by endogenous and exogenous factors. Understanding the relationship between the composition of the oral microbiota and the development of dental and somatic issues can lead to the development of new technologies for both prevention and treatment.

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ASSESSMENT OF THE PREVALENCE AND SEVERITY OF PERIODONTAL DISEASES IN THE ADULT POPULATION OF THE STAVROPOL REGION

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ОЦЕНКА РАСПРОСТРАНЕННОСТИ И ТЯЖЕСТИ ЗАБОЛЕВАНИЙ ПАРОДОНТА У ВЗРОСЛОГО НАСЕЛЕНИЯ СТАВРОПОЛЬСКОГО КРАЯ

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According to the World Health Organization, periodontal diseases represent one of the most prevalent global health issues, affecting nearly 88 % of the population. This study assessed the prevalence and severity of periodontal pathology in 387 residents of the Stavropol Region using the Community Periodontal Index. Findings revealed a high burden of periodontal disease, with both severity and prevalence increasing with age, peaking in individuals aged 45–54. In older