Research Article

Identification of Age and Gender Specific Bacteria in Human Saliva through Next- Generation Sequencing

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How to cite this article: Shubham Yadav, Vijay Tripathi, Vaibhav Saran. Identification of Age and Gender Specific Bacteria in Human Saliva through Next- Generation Sequencing. Indian Journal of Forensic Medicine and Toxicology 2023;17(4).

Abstract

Characterization of human saliva through Next-Generation Sequencing (NGS) has emerged as a valuable tool for understanding the complex microbial communities residing in the oral cavity. This study aims to investigate the age and gender-based variations in the salivary microbiome using NGS technology. Saliva samples were collected from a diverse population representing females among below 45 Vs above 45 and males among below 45 Vs above 45. The DNA from the samples was extracted, and theV3-V4 region of the 16S rRNA gene was amplified for NGS analysis. The obtained sequences were processed and analysed using bioinformatics tools to determine the microbial composition and diversity. Preliminary results revealed distinct microbial profiles, indicating the potential influence of age and gender on the salivary microbiome.

Further research is warranted to explore additional factors and expand the scope of habit-based analysis in saliva-based microbial characterization through NGS.

Keywords: Saliva, Microbial Diversity, Next Generation Sequencing, DNA Metabarcoding, Microbial Forensic

Introduction

Saliva: Saliva is a clear, watery fluid that is produced by the salivary glands in the mouth. It plays a crucial role in the process of digestion and maintaining oral health. Saliva contains enzymes that help break down food and initiate the digestion process [1]. In addition to its digestive functions, saliva has other important roles. It helps to keep the mouth clean by washing away food particles and bacteria, which can help prevent tooth decay and gum disease. Saliva also contains antibodies and antimicrobial compounds that help fight against harmful bacteria and viruses, contributing to the overall health of the oral cavity [2]. The production of saliva is primarily controlled by the autonomic nervous system, which means it happens involuntarily and is influenced by factors such as the sight, smell, or even the thought of food. Overall, saliva is an essential fluid that plays a vital role in digestion, oral health, and maintaining a comfortable and functioning mouth [3, 4].

Salivary Microbiome: The salivary microbiome refers to the community of microorganisms, including bacteria, viruses, fungi, and other microbes, that inhabit the saliva in the oral cavity. Like other parts

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of the human body, the mouth has its own unique microbial ecosystem, and saliva acts as a reservoir for these microorganisms[5–8]. The composition of the salivary microbiome is influenced by various factors, including genetics, oral hygiene practices, diet, smoking, medications, and overall health. It is a dynamic and complex ecosystem with a diverse range of microbial species. While the exact composition of the salivary microbiome can vary between individuals, certain bacterial species are commonly found.Some of the bacterial genera commonly found in the salivary microbiome include *Streptococcus, Neisseria, Veillonella, Haemophilus, and Prevotella,* among others. These bacteria can have both beneficial and pathogenic effects on oral health. Beneficial bacteria can help maintain oral health by competing with harmful bacteria for resources and producing antimicrobial substances. On the other hand, certain pathogenic bacteria, such as *Streptococcus* mutans, can contribute to tooth decay and gum disease[9,10].

Research on the salivary microbiome has revealed its potential role in oral and systemic health. Imbalances or dysbiosis in the salivary microbiome have been associated with various oral conditions, including dental caries (tooth decay), periodontal disease (gum disease), and oral infections[11-14]. Studying the salivary microbiome can provide valuable insights into oral health and disease, and it may pave the way for the development of new diagnostic and therapeutic approaches[15]. However, it's important to note that the field of salivary microbiome research is still relatively new, and further studies are needed to fully understand the complex interactions between the oral microbiome and human health.

Role of Metabarcoding in Forensics:

Metabarcoding is a molecular technique that involves the simultaneous amplification and sequencing of multiple DNA regions to identify and characterize the species present in a sample[16]. In the field of forensics, metabarcoding has the potential to play a significant role in various applications and here are a few examples:

a) Forensic Entomology: Metabarcoding can aid in determining the species of insects found on a crime scene or associated with a deceased individual. By analysing the DNA extracted from insect samples, forensic entomologists can identify the insect species present, estimate the post-mortem interval, and provide valuable information for criminal investigations.

b) Wildlife Forensics: Metabarcoding can be employed to analyse DNA samples collected from various wildlife-related forensic investigations. This can include the identification of species from illegally traded products (such as wildlife parts or products made from endangered species), tracking the origin of seized animal products, and identifying the presence of specific species in forensic samples (e.g., animal hair, feathers, or faeces) found at crime scenes.

c) Environmental Forensics: Metabarcoding can assist in environmental forensic investigations, where the identification of species present in an environment or water body is required. It can be used to assess biodiversity, monitor invasive species, identify the source of environmental contamination, and provide evidence in cases involving illegal dumping or pollution.

d) Human Forensics: Metabarcoding has the potential to contribute to human identification in forensic contexts. For example, it can be used to analyse DNA samples recovered from crime scenes, including mixed DNA samples, degraded samples, or samples with limited genetic information. Metabarcoding can provide a broad survey of species present in the sample, potentially identifying trace amounts of DNA from suspects or individuals of interest.

It's important to note that while metabarcoding holds promise in forensic applications, it is still an evolving field, and its implementation in forensic laboratories requires careful validation, standardization, and adherence to established protocols. Additionally, the interpretation of metabarcoding results requires expertise in bioinformatics and the comparison of DNA sequences with comprehensive reference databases[17-19].

Nonetheless, metabarcoding has the potential to enhance the capabilities of forensic investigations by providing valuable information about species present in a sample, thereby aiding in criminal investigations and legal proceedings.

Methodology

Sampling: The participants were advised to clean their teeth in the morning and then fast for one hour before the sample collection. Spitting into a sterile tube method was used to collect saliva samples. To avoid any changes in the bacterial ecology, samples were kept at 20 degrees Celsius until analysis.

DNA extraction and amplification: The bacterial DNA were isolated using Himedia "HiPurA® Forensic Sample Genomic DNA purification Kit" from Saliva Sample. [20,21] This system uses the HiElute Miniprep Spin Column, and the main steps are as follows:

a) Saliva samples were transferred onto 0.5cm2 areas of Filter paper and cut it into smaller pieces. The pieces were transferred into Eppendorf tubes.

b) 300 µl Lysis Solution AL (DS0015), 20 µl Proteinase K (20 mg/ml) and 20 µl of 1M DTT were added to the material in the collection tube. It was mixed thoroughly by pulse-vortexing for 10-15 seconds. Then the tubes were incubated at 55°C for 1½ hours in a dry hot water bath. The tubes were vortexed after every 10 minutes for 10-15 seconds to improve lysis of the sample material.

c) 300 µl of Lysis Solution C1 (DS0010) was added to the same tube and mixed all the reagents thoroughly by pulse-vortexing for 10- 15 seconds. The tubes were then incubated at 70°C for 10 minutes in a dry hot water bath and were vortexed after every 3 minutes for 10-15 seconds to improve lysis of the sample material. The tubes were then centrifuged at 12,000-16,000 x g (≈13,000-16,000 rpm) for 1 minute at room temperature.

d) The supernatant obtained after centrifugation was transferred carefully into a new 2.0 ml collection tube and 300 µl of ethanol (96- 100%) was added for preparation of the lysate for binding. The reagents were mixed thoroughly by vortexing for 5-10 seconds.

e) Using a wide bore pipette tip, 650 µl of the mixture (including any precipitate, which may have formed) was added to the HiElute Miniprep Spin Column (Capped). The tubes were centrifuged for 1 minute at 6000 g (≈8000 rpm) at room temperature. The flow-through liquid was discarded. The steps were repeated with the remaining sample and the flow-through liquid was discarded.

f) The column was placed in a same 2.0 ml collection tube and 700 µl of diluted Prewash Solution (PW) (DS0011) was added to the column. The tubes were centrifuged at 6,500 x g (≈10,000 rpm) for 1 minute at room temperature (15-25°C). The flow-through liquids were discarded. Re-used the same collection tube with the column.

g) Another 700 µl of diluted Wash Solution (WS) (DS0012) were added to the HiElute Miniprep Spin Column (Capped) and was centrifuged at 12,000-16,000 x g (≈13,000-16,000 rpm) for 1 minute at room temperature. The flow-through liquid was discarded and the same collection tube was reused. The column was centrifuged for 2 minutes at 20,000 x g (≈14,000 rpm) to dry the column membrane (to remove the traces of residual ethanol, if observed). The column was then placed in a new uncapped 2.0 ml collection tube.

h) 30 µl of the Elution Buffer (ET) (DS0040) was added directly onto the center of the HiElute Miniprep Spin Column (Capped) membrane without spilling to the sides. The tubes were allowed for incubation for 1 minute at room temperature. The tubes were centrifuged at [3]6,500 x g (≈10,000 rpm) for 1 minute to elute the DNA. The eluates were transferred to a fresh capped 2ml collection tube for longer DNA storage.

The eluate contains pure genomic DNA. For short-term storage (24-48 hrs.) of the DNA, 2-8°C is recommended. For long-term storage, -20°C or lower temperature (-80°C) is recommended.

i) Extracted DNA from the samples were subjected to Nano Drop and GEL Check before being taken for PCR amplification. The Nano Drop readings of 260/280 at an ~ value of 1.8 to 2 is used to determine the DNA's quality.

j) Composition of TAQ Master MIX:
   i) High-Fidelity DNA Polymerase
   ii) 0.5mM dNTPs
   iii) 3.2mM MgCl2
   iv) PCR Enzyme Buffer

Primer Details:

16sF: 5'AGAGTTTGATGMTGGCTCAG3'

16sR: 5' TTACCGCGGCMGCSGGCAC3'

Conditions used: 40ng of Extracted DNA is used for amplification along with 10pM of each primer with the following condition: Denaturation at the 95°C for 15 seconds, Annealing at the 60 °C for 15 seconds, Elongation at the 72 °C for 2 mins. Final Extension at 72 °C for 10 mins and hold at 4°C.

Overview of Sequencing Protocol: To produce the sequencing libraries, the amplicons from each sample were filtered with Ampure beads to eliminate unneeded primers, and an additional 8 cycles of PCR were done using Illumina barcoded adapters. Ampure beads were used to purify the libraries, and the Qubit dsDNA High Sensitivity assay kit was used to quantify them. Illumina Miseq with 2x300PE v3 sequencing kit was used for the sequencing.

Bioinformatics protocol: FASTQC and MULTIQC are used for raw data QC, followed by TRIMGALORE for adapter and low-quality read trimming. MOTHUR processes are used to process the trimmed reads, which include paired end read merging, chimera elimination, and OUT abundance computation and estimation correction. This approach permits highly accurate genus-level research.

SILVA, GREENGENES, and NCBI databases were used. Each read is categorised based on its percentage coverage and identification. The 16S approach proved helpful in identifying bacteria in a mixed sample as well as analyzing the nature of a bacterial community.

Flash: The second step was to overlap all the paired reads to produce the consensus sequences for each sample. FLASH (Fast Length Adjustment of Short reads) was the chosen software to correctly overlap the reads, specifically version 1.0.2 was used. FLASH 1.0.2 was run locally using the following command line and parameters (GG4 R1 and GG4 R2 are the two individual reads that are to be paired together).

Results and Discussion

The Results generated post bioinformatics analysis gave the microbial diversity in human saliva with different age and gender, the graphical representation of microbial diversity in females among below 45 Vs above 45 and males among below 45 Vs above 45 are shown from the following figures and a comparative chart for unique bacteria respective to their habits were identified. It is evident from the results that age and gender has a significance on the bacterial diversity as well as few bacteria are specific to age and gender of any individual as they get optimum growth conditions related to these factors.

Top 10 bacterial diversity in female among below 45 and above 45 volunteers:

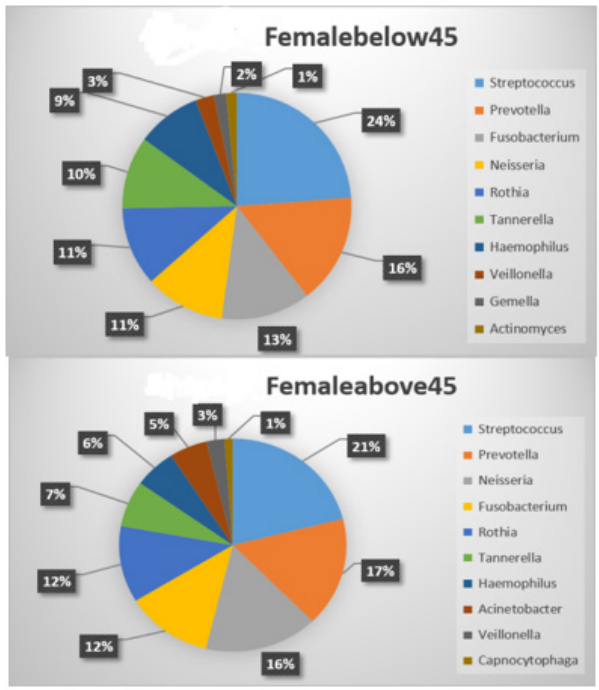

Fig. 1 Genus wise distribution among Female below 45 and above 45 of age

Females below 45 of age had higher concentrations of *Streptococcus, Neisseria,* and *Haemophilus,* and lower concentrations of *Prevotella, Neisseria, Rothia, Veillonella,* and *Gemella* when compared with Females above 45 of age as shown in Figure 1. However, some bacteria were unique to both the categories, for below 45 of age, *Actinomyces* and for above 45 of age, *Acinetobacter* and *Capnocytophaga.*

Table 1: Analysis of bacterial specific diversity among Female Below 45 and above 45

Analysis of bacterial Diversity	
Female below 45	**Female above 45**
1. Akkermansia	1. Aeromonas
2. Arcobacter	2. Butyrivibrio
3. Bacillus	3. Cellulomonas
4. Bosea	4. Clostridioides
5. Butyrivibrio	5. Clostridium
6. Cardiobacterium	6. Comamonas
7. Cellulomonas	7. Corynebacterium
8. Chloroflexus	8. Delftia
9. Collinsella	9. Desulfovibrio
10. Curtobacterium	10. Dichelobacter
11. Gemmata	11. Dyadobacter
12. Helicobacter	12. Ezakiella
13. Mesorhizobium	13. Hydrogenophaga
14. Micavibrio	14. Lactobacillus
15. Niabella	15. Lactococcus
16. Paludisphaera	16. Lautropia
17. Pelolinea	17. Mesorhizobium
18. Staphylococcus	18. Morganella
	19. Novosphingobium
	20. Pediococcus
	21. Proteus
	22. Rhizobium
	23. Shewanella
	24. Sphingorhabdus

Top 10 bacterial diversityin Male above 45 and below 45 volunteers:

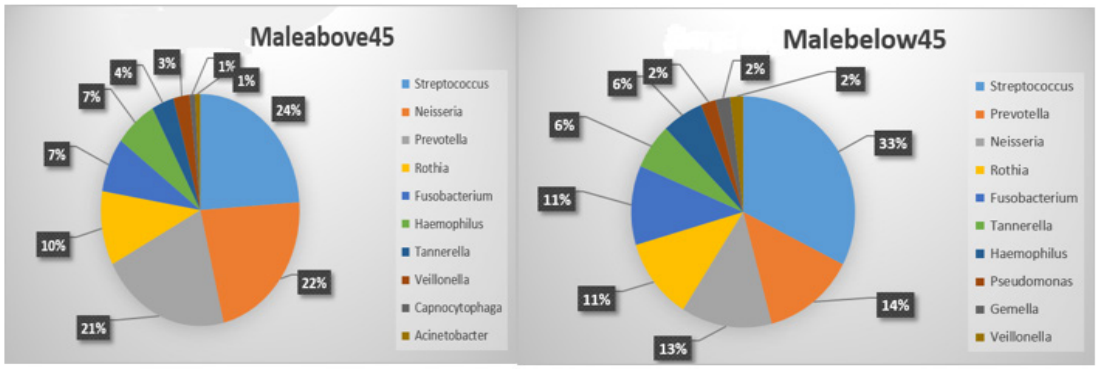

Fig. 2 Genus wise distribution among Male above 45 and below 45 of age

Males below 45 of age had higher concentrations of *Streptococcus, Rothia,* and *Fusobacterium,* and lower concentrations of *Prevotella, Neisseria,* and *Haemophilus* when compared with Males above 45

of age as shown in Figure 2. However, some bacteria were unique to both the categories, for below 45 of age, *Tannerella, Gemella* and *Pseudomonas* and for above 45 of age, *Acinetobacter* and *Capnocytophaga.*

Table 2: Unique bacterial diversity among male below 45 and above 45

Analysis of Bacterial Diversity	
Male below 45	**Male above 45**
1. Achromobacter	1. Citrobacr
2. Bergeyella	2. Erythror
3. Blastochloris	3. Mycoplasma
4. Chitinophaga	4. Sorangium
5. Chryseolinea	5. Tessaracoccus
6. Citrobacter	6. Xanthomonas
7. Comamonas	
8. Devosia	
9. Methylobacterium	
10. Moraxella	
11. Olsenella	
12. Ruminococcus	
13. Serratia	
14. Slackia	
15. Sphaerotilus	
16. Sphingorhabdus	
17. Streptobacillus	

Discussion

The findings of the study suggest that there are significant differences in the salivary microbiomes between females below 45 years of age and females above 45 years of age. Specifically, females below 45 years of age had higher concentrations of Streptococcus, Neisseria, and Haemophilus, while having lower concentrations of Prevotella, Neisseria, Rothia, Veillonella, and Gemella compared to females above 45 years of age.

These results are in line with previous research that has documented changes in the oral microbiota of females with respect to age. One study by Kumar in 2020[22]investigated the impact of female sex hormones on periodontal bacteria[22]. They found that hormonal fluctuations in women throughout their reproductive life can influence the composition of the oral microbiome, leading to changes in bacterial species abundance. Although the specific bacteria mentioned in the study may not be directly addressed, it provides a context for understanding the influence of age-related hormonal changes on the oral microbiota in females.

Regarding males, the study found that males below 45 years of age had higher concentrations of Streptococcus, Rothia, and Fusobacterium, and lower concentrations of Prevotella, Neisseria, and Haemophilus compared to males above 45 years of age. These results are consistent with other studies that have demonstrated the impact of age on the oral microbiota in males.

A study by Li in 2000 investigated systemic diseases caused by oral infections and reported changes in the oral microbiota associated with age[23]. They found that age-related variations in the oral microbiome could contribute to the development of systemic diseases. While the specific bacteria mentioned in the study may differ, it suggests that age-related changes in the oral microbiota of males can have implications for overall health.

Conclusion

In conclusion, the identification of salivary bacteria through new generation sequencing has provided valuable insights into age and gender specific bacterial species. This research has demonstrated that certain bacterial species are uniquely associated with age and gender. The ability to profile salivary bacterial DNA based on these factors has promising applications in the field of forensics, as it helps to narrow down the pool of suspects in forensic investigations. However, further studies and validation are required to ensure the reliability and accuracy of this technique. Establishing comprehensive databases of age and gender specific bacterial profiles would facilitate effective comparison and identification of suspects. Despite challenges, salivary bacterial DNA profiling it opens up new avenues for gathering evidence and linking individuals, thereby aiding in the identification and narrowing down of suspects. As technology continues to advance, the field of forensic microbiology holds great promise for enhancing the investigative capabilities of law enforcement agencies and ultimately ensuring justice is served.

Conflict of interest: We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Ethical Clearance: The author wishes to inform that all human ethical clearance were taken from the Ethical Committee of the University for collection of human saliva samples and conducting the experiments. The samples were also taken post duly signed consent form by the volunteers.

References

1. Roblegg E, Coughran A, Sirjani D. Saliva: an all-rounder of our body. Eur J Pharm Biopharm. 2019;142:133-41, ISSN 0939-6411. doi: 10.1016/j.ejpb.2019.06.016, PMID 31220573.
2. Mandel ID. The functions of saliva. J Dent Res. 1987;66(1_suppl):623-7. doi: 10.1177/00220345870660S103.
3. Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. Saliva diagnostics – current views and directions. Exp Biol Med (Maywood). 2017;242(5):459-72. doi: 10.1177/1535370216681550, PMID 27903834.
4. Lee Y.H., Wong D.T. Lee Y.H. and W. D.T. Saliva: an emerging biofluid for early detection of diseases. Am J Dent. 2009 Aug;22(4):241-8. PMID 19824562, PMCID PMC2860957.
5. Rasiah IA, Wong L, Anderson SA, Sissons CH. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol. 2005;50(9):779-87. doi: 10.1016/j.archoralbio.2005.02.001, PMID 15970209.
6. Rasiah IA, Wong L, Anderson SA, Sissons CH. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol. 2005;50(9):779-87. doi: 10.1016/j.archoralbio.2005.02.001, PMID 15970209.
7. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res. 2012;22(11):2146-52. doi: 10.1101/gr.140608.112, PMID 23064750.
8. Yamanaka W, Takeshita T, Shibata Y, Matsuo K, Eshima N, Yokoyama T et al. Compositional Stability of a Salivary Bacterial Population against supragingival microbiota Shift following Periodontal Therapy. PLOS ONE. 2012;7(8):e42806. doi: 10.1371/journal.pone.0042806, PMID 22916162.
9. Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T et al. Biogeography of the ecosystems of the healthy human body. Genome Biol. 2013;14(1):R1. doi: 10.1186/gb-2013-14-1-r1, PMID 23316946.
10. Goodson JM, Groppo D, Halem S, Carpino E. Is obesity an oral bacterial disease? J Dent Res. 2009;88(6):519-23. doi: 10.1177/0022034509338353, PMID 19587155.
11. Piombino P, Genovese A, Esposito S, Moio L, Cutolo PP, Chambery A et al. Saliva from obese individuals suppresses the release of aroma compounds from wine. PLOS ONE. 2014;9(1):e85611. doi: 10.1371/journal.pone. 0085611.
12. Yoshizawa JM, Schafer CA, Schafer JJ, Farrell JJ, Paster BJ, Wong DTW. Salivary biomarkers: toward future clinical and diagnostic utilities. Clin Microbiol Rev. 2013;26(4):781-91. doi: 10.1128/CMR.00021-13, PMID 24092855.
13. Li K, Bihan M, Methé BA. Analyses of the stability and core taxonomic memberships of the human microbiome. PLOS ONE. 2013;8(5):e63139. doi: 10.1371/journal.pone.0063139, PMID 23671663.
14. De Filippis F, Vannini L, La Storia, A., Laghi, L., Piombino, P., Stellato, G., … Ercolini, D. (2014). The Same Microbiota and a Potentially Discriminant Metabolome in the Saliva of Omnivore, Ovo-Lacto-Vegetarian and Vegan Individuals. PLoS ONE, 9(11), e112373. doi:10.1371/journal.pone.0112373.
15. Li J, Quinque D, Horz HP, Li M, Rzhetskaya M, Raff JA et al. Comparative analysis of the human saliva microbiome from different climate zones: Alaska, Germany, and Africa. BMC Microbiol. 2014;14(1):316. doi: 10.1186/s12866-014-0316-1, PMID 25515234.
16. Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. PLOS ONE. 2012;7(6):e34242. doi: 10.1371/journal.pone.0034242, PMID 22719824.
17. Ventura Spagnolo E, Stassi C, Mondello C, Zerbo S, Milone L, Argo A. Forensic microbiology applications: a systematic review. Leg Med (Tokyo). 2019;36:73-80. doi: 10.1016/j.legalmed.2018.11.002, PMID 30419494.
18. Prescott SL, Larcombe DL, Logan AC, West C, Burks W, Caraballo L, et al. The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. World Allergy Organ J. 2017;10(1):29. doi: 10.1186/s40413-017-0160-5, PMID 28855974.

19. D'Angiolella G, Tozzo P, Gino S, Caenazzo L. Trick or treating in forensics – the challenge of the saliva microbiome: a narrative review. Microorganisms. 2020;8(10):1501. doi: 10.3390/microorganisms8101501, PMID 33003446.

20. Procopio N, Lovisolo F, Sguazzi G, Ghignone S, Voyron S, Migliario M, et al. 'Touch microbiome' as a potential tool for forensic investigation: a pilot study. J Forensic Leg Med. 2021;82:102223. doi: 10.1016/j.jflm.2021.102223, PMID 34343925.

21. Birren B, Lai E. Pulsed field gel electrophoresis. Vol. 1993. Academic press, Isbn 9780121012908; 1993. p. iii.

22. Bennett ST, Barnes C, Cox A, Davies L, Brown C. Toward the $1000 human genome. Pharmacogenomics. 2005;6(4):373-82{382. doi: 10.1517/14622416.6.4.373.

23. Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic diseases caused by oral infection. Clin Microbiol Rev. 2000 Jan;13(4):547-58. doi: 10.1128/CMR.13.4.547, PMID 11023956.