Humboldt Journal of Microbiology

Volume 23 Article 3

2023

Contrast of Smokeless Tobacco and it's Microbiological Footprint in Users and Non-Users

Monayjia Brown
Cal Poly Humboldt, mab272@humboldt.edu

Katie Robbins
Cal Poly Humboldt, kar161@humboldt.edu

Elyse McKinney
Cal Poly Humboldt, ejm122@humboldt.edu

Sydney Snell
Cal Poly Humboldt, sss127@humboldt.edu

Humboldt Journal of
Microbiology
2023, Volume 23

Contrast of Smokeless Tobacco and it's Microbiological Footprint in Users and Non-users

Sydney Snell, Katie Robbins, Elyse McKinney, Monayjia Brown

ABSTRACT

Smokeless tobacco products such as vapes have gained significant popularity over the last 20 years (7), and it is clear that the effects of these products are primarily unknown. This study examines how the microbial profiles found in smokeless tobacco products, such as vapes, can be used in forensic science. By linking these profiles to their owners, investigators can identify suspects in cases where traditional evidence is lacking. This information could provide valuable assistance in tracking victims and matching perpetrators or victims to crime scenes. Forensic science involves any evidence found at a crime scene that is examined and analyzed to expand on the findings. Forensic science can be defined as applying a range of sciences to criminal and civil laws. Forensic science is crucial in criminal investigation, but it is not known for using microbiology to create a specific biological profile (8). This research could break microbiology into the field of forensic science by using the example of creating microbial footprints to help with other instances in the field. We will analyze the bacteria count in tobacco products and users' microbiomes. Using TSA and Blood agar plates, comparisons between bacteria abundance and diversity in users and non-users will be quantified. We will identify differences in microflora between smokeless tobacco users and non-users. The bacteria produced by users significantly differed from those produced by non-users due to the vapors taken in from the smokeless tobacco product. Vaping growth does not transfer to smokeless tobacco, making it difficult to identify users (2). The microflora on the product can help researchers study its impact on the human microbiome, but forensic profiling is not possible due to the lack of transference from the smokeless tobacco product to the users hands and nose.

INTRODUCTION

Over the past 20 years, society has seen a rise in smokeless tobacco products (8). These include smokeless tobacco products such as: dry snuff, snus, zyns, vapes, and chewing tobacco. While it is known that different smokeless tobacco products harbor varying bacterial microbiota and varying levels of culturable bacteria (6), this project mainly focuses on the smokeless tobacco products, ELF BARS. Smokeless tobacco products have become more disposable than cigarettes in the past five years. In the last decade, there has been an epidemic affecting over 300 million people worldwide involving smokeless tobacco and its effects (1,4). Smokeless tobacco products such as "ELF BARS" and "Flumes" have gained enormous popularity among young people, such as high schoolers and college kids. "ELF BARS and Flumes" are two different, highly popular brands of disposable smokeless tobacco products, not only in Humboldt County but in all of California. Smokeless tobacco products have become the new and improved cigarette, yet scientists and researchers do not know much about their effects. What can be found in the little amount of time that these products have been around and gained popularity is that they are easily accessible, incredibly disposable, and most likely detrimental to your health if they are anything similar to cigarettes.

This research outlines the Microbiology of oral bacteria and its changes found in vaping among anonymous users focusing specifically on one type of smokeless tobacco, which has been determined to be "Elf Bars." Sticking to one smokeless tobacco product eliminates any other mechanisms that might come into play, such as different warehouses, different smokeless tobacco product materials, and where they were made. Much of this research and information found will be helpful in areas of Forensic science (9). Forensic science is crucial in criminal investigation as it entails any evidence found at a crime scene that is examined and analyzed to expand on other findings involved. Using scientific methods to investigate and solve a crime with the use of microbial profiles, let alone microbiology in general in its practice, has been underdeveloped as of modern times. Forensic science could use something like this when conducting assays on crime scenes to determine an outcome or find a perpetrator based on their microbial

profile. In the forensic field, microbial profiles could help differentiate people and even their objects, in this instance, their smokeless tobacco products. Being able to extract someone's bacteria profile from their mouths, hands, and noses creates a print for each person getting tested and could be used similarly to a fingerprint database. Knowing the specific bacteria found in one person's mouth compared to another will help create these profiles that will allow them to be compared and matched to the profile of the smokeless tobacco product. Having more than one dataset of bacteria like the hands, mouth, and nose allows for an easier match to the smokeless tobacco product.

It will be hypothesized that the microbiome found in smokeless tobacco products can be matched with its specific user by comparing it to the human microbiome. This research will present microflora found in the mouths, hands, and noses of people who use smokeless tobacco products and people who do not. This will then be compared to the individual smokeless tobacco products and highlights the different microflora in these products. This data can all be used to create different microbiological fingerprints of people who use smokeless tobacco products and differentiate them from non-users. This project hopes to use the microbiological fingerprint created by smokeless tobacco products and from people to match the 'ELF Bar' to their respective users. This will show an effect on the human microbiome and change of mouth and nasal flora due to the use of smokeless tobacco. This research will also prove the need for microbial profiles and microbiology to be used more extensively in forensic science.

METHODS

Samples were collected from four individuals: two of whom were ELF bar users and two who were non-users. Samples were taken in the microbiology lab at Cal Poly Humboldt throughout the month of April 2023. The subjects used sterile cotton swabs to collect samples from their mouths using about 1mL of diluted and plated spit. The subjects also swabbed their noses and hands; this will be collected and grown on blood agar plates at 37 C for 48 hours. We quantified several colony morphologies as a metric of diversity and the total number of colony-forming units (CFU) as a metric of abundance. The smokeless tobacco products belonging to the two ELF BAR users will be swabbed and placed onto blood agar plates. Blood agar plates were used because they contain a rich source of nutrients supporting the growth of many bacteria.

We swabbed the areas around the product and the mouthpiece of the ELF bar to test for hand-transferred microorganisms and potential bacterial contamination in smokeless tobacco products to the user's mouth. First, we swabbed the areas around the product where the user's hands typically come into contact. Next, swabbing the mouthpiece of an unused or unopened smokeless tobacco product as a control. Then, carefully transfer the collected samples onto agar plates for culture and analysis. This process will help identify any bacteria on the product and can aid in determining potential health risks associated with its use. Both blood agar and TSA plates will be placed inside the incubator for 2-3 days at 37 degrees C.

After incubation, quantification of abundance and diversity will take place, number of morphology types, along with how many morphological types there were of each, will be taken into account. Then each morphology type found on smokers' and non-smokers mouth dilution plates will be taken using a sterile loop and stained using the gram stain technique. This will allow us to differentiate morphotype into another category which is either gram positive or gram negative. This will show a difference when conducting statistical analysis and creating figures between smoker and non-smoker abundance and diversity. This allows for a more extensive microbial fingerprint, being able to categorize which morphotypes are gram positive or negative allows a difference to be shown in smoker and non-smoker bacteria. Diversity was calculated from each individual's mouth by using about 1ml of spit from each subject and diluting it from the stock concentration 10^6, then observing the TSA plates with about 30-100 CFU. Then a gram stain was applied to each of the different bacteria morphotypes.

We compared the results with the unopened smokeless tobacco product samples to the ELF BAR users' samples, using morpho type along with the number of colonies accumulated between the two smokeless tobacco product users. The samples from the control subjects will be compared to the ELF BAR users to show a significant difference between the two. We compared the morphotype diversity of users and non-users using an ANOVA. We ran a Chi-Square analysis test to see the statistical significance between the difference in the microflora of the users and non-users and the bacteria on the smokeless tobacco products.

Figures such as a bar graph were created to show the distinction between these two identities using mean and standard deviation. The purpose was to visually compare and

contrast the data and make it easier to interpret. The bar graph allowed us to display the information in a clear and organized way, highlighting any significant differences between the two identities being studied. By these methods and figures below, we were able to communicate the findings of this research effectively.

Statistical Analysis

Chi-Square Analysis

The Chi-Square test was used to find the significant difference between gram-positive and gram-negative bacteria in smokers and non-smokers. The results showed that we could accept the idea that there is an association between how much gram-positive and gram-negative bacteria are found in smokers and non-smokers (P 0.04). In this instance, there is a high amount of Gram-positive bacteria in the smokers which is significantly different from both the gram-positive and gram-negative bacteria found in non-smokers.

T-test

A T-test was run on the diversity numbers taken from the dilution plates of the subjects' mouths and separated between smokers and non-smokers. The T-test value that was obtained was(0.77). This value is close to 1, which helps determine that this is a high T-test value. Higher values on a T-test indicate that there are large differences between the two sample sets it was run on. This can help conclude that the diversity numbers obtained between smokers and non-smokers are significantly different from each other and that smokers have a difference in diversity compared to non-smokers.

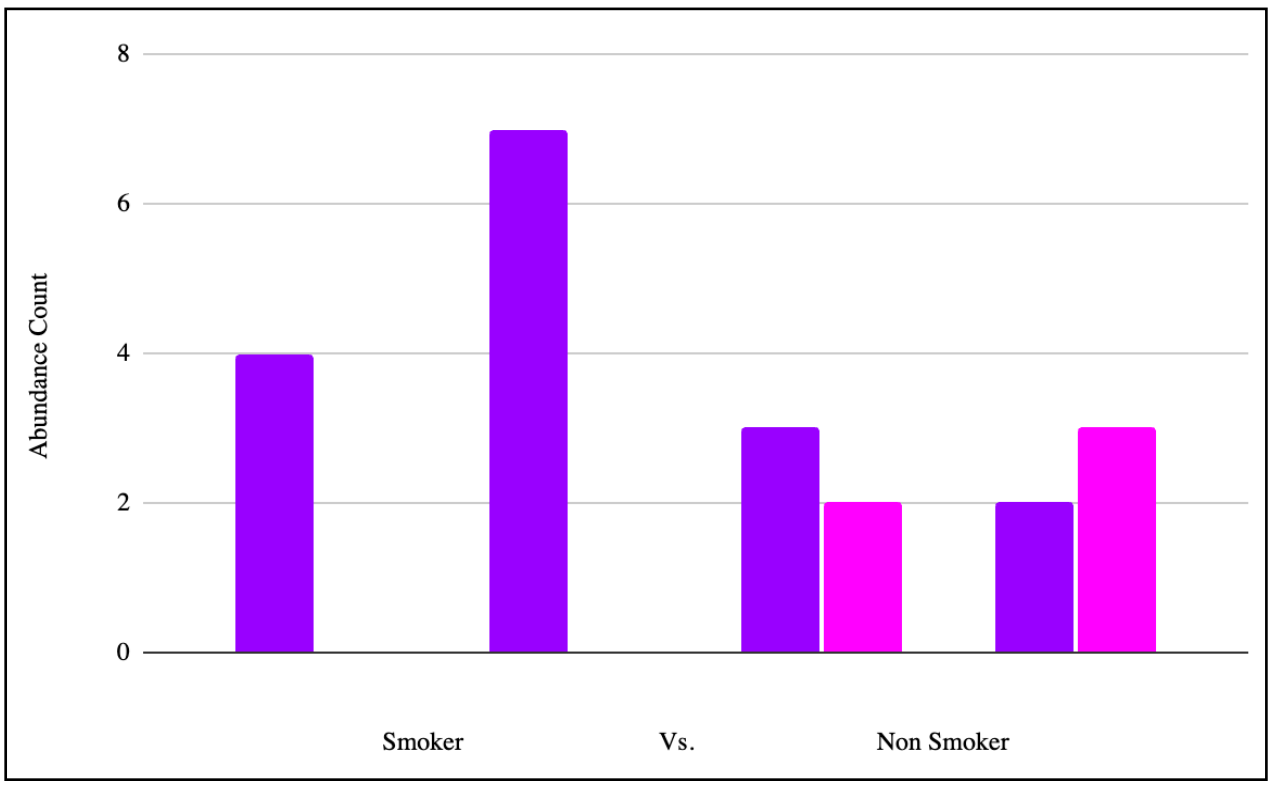
One way ANOVA test

A one way ANOVA test was run on the data gathered from the abundance of gram-negative and gram-positive bacteria found in the subjects' mouths. The test revealed that there is a significant difference in these bacteria between smokers and non-smokers (P 0.04). The staggering difference between both smokers only having gram-positive bacteria while both non-smokers had an even distribution of both. This finding could help with creating criteria for microbial profiles in forensics, if it becomes a pattern that smokers typically only have gram positive bacteria found in their mouths, there may be a chemical element discharged from the smokeless tobacco product causing this. Since, normally a healthy human mouth has both gram negative and gram positive bacteria.

RESULTS

The mean morphotype diversity was very similar between users and non-users; the standard deviation in users was much larger than compared to non-users (Figure 2). Non-smokers had the same diversity of morphotypes, while smokers had variable levels of diversity. Perhaps, the use of smokeless tobacco

Figure 2.
Abundance of Gram Negative and Gram Positive bacteria found in mouths.



products is the cause of this variability. All morphotypes of the ELF bar users were gram-positive. In contrast, the non-smokers had a healthy abundance of gram-negative and gram-positive bacteria (Figure 2). By analyzing samples both microbiologically and forensically, it is possible to distinguish between a smoker and a non-smoker with these results. The results of this study suggest that vaping devices may significantly influence the microbial communities present in individuals' oral and nasal cavities. The morphotypes observed in smokers differed from those in non-smokers, suggesting a potential effect of vaping on oral and nasal microbiota.

Counts of different morphologies were taken from mouth bacteria, and only two morphologies overall were recorded for each variable. This supports that the mouths, compared to the two other variables of hands and nose, have the most bacteria. Non-smokers and smokers showed a wide range of morphotypes. Smokers portrayed distinct morphotypes, suggesting a potential impact of vaping on oral and nasal microbiota. (Figure 3). The data collected shows higher average levels of diversity for smokers compared to non-smokers and is primarily related to mouth bacteria. This supports the conclusion that the study should focus on the mouth microbiome. Since the mouth and the smokeless tobacco are transferred to one another more than the other bacteria of the hands and nose. Since neither bacteria was found on both the smokeless tobacco product and the hands and the nose, leading to the assumption of no transference (Table 1).

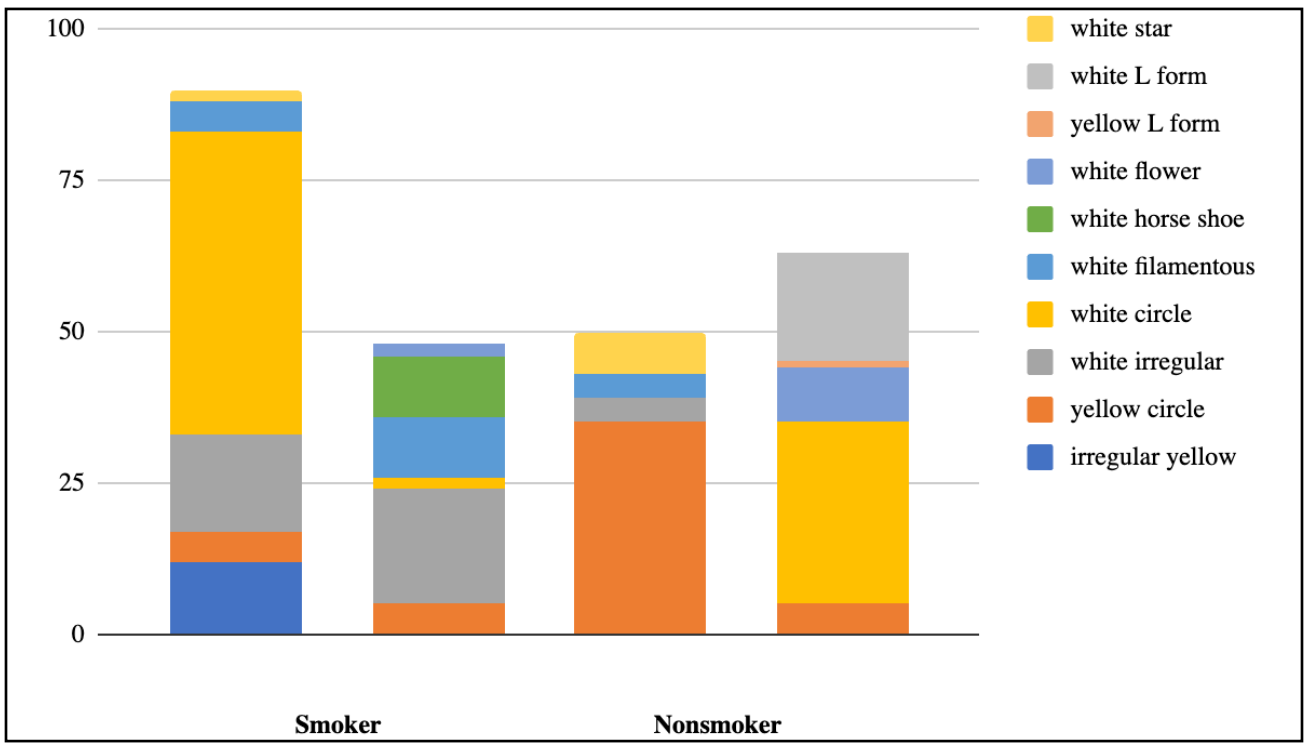
DISCUSSION

Smoking from ELF BARs ignites a cascade of vapor that allows for direct contact with a user's mouth since that is the pathway that transfers the smoke product to the lungs and back. From the studies before this research, it has been known that vaping affects users in some way, just like cigarettes have been found to affect users' oral microbiomes (1). This

Table 1.
Table of diversity for smokers and non-smokers.

Variable	Smokers Means	NonSmokers Means	Smokers SD	NonSmokers SD
M	725	363.5	318.1980515	152.027958
N	363	45	193.747258	63.63961031
H	124.5	115.5	108.1873375	50.20458146

Figure 3.
Abundance of different morphology types found between smokers and non-smokers.

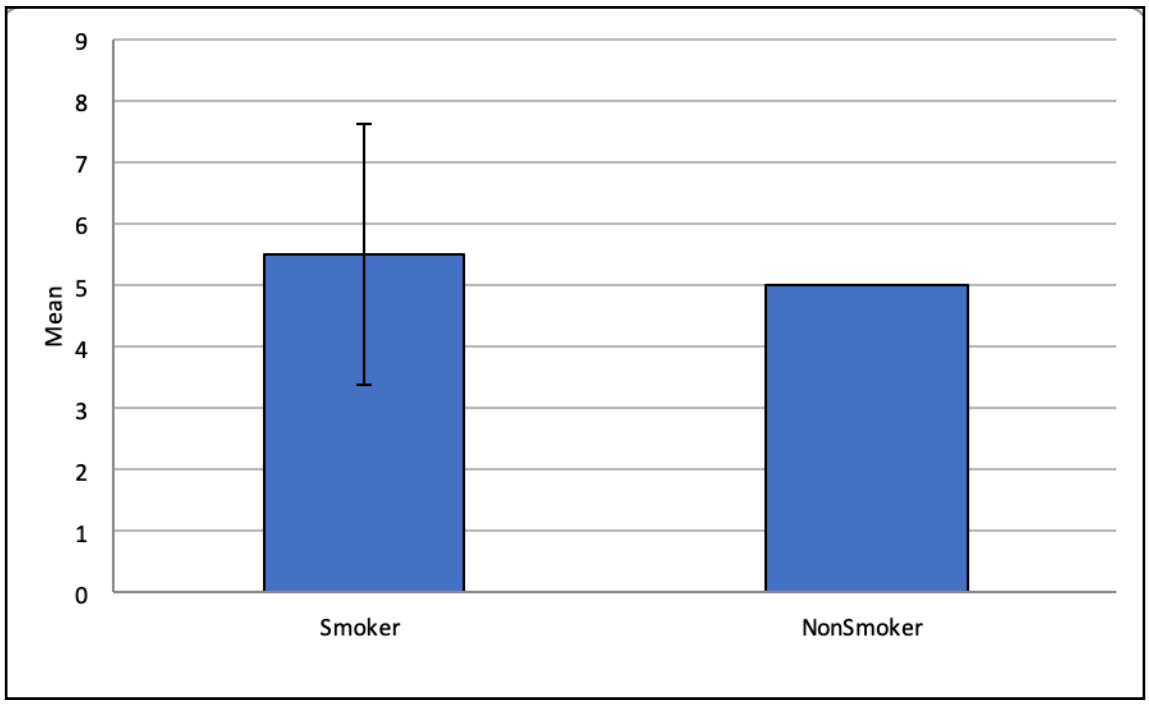


study focuses on the mouth and the microbiome and what can be transferred to the ELF BAR or what bacteria could be transferred from the ELF BAR to the users' mouth. ELF BARS and other smokeless tobacco products are known to contain dozens of chemicals, some of which are toxic, and disrupt the natural oral bacteria (5). The smokeless tobacco users only had gram-positive stains from all morphotypes of bacteria, which could conclude that the microbiome is affected by the vapor, and only the positive bacterium has resistance, as well as the nicotine contents affect the users' healthy oral microbiome (2). This could have important implications for developing certain oral health conditions with the continued use of smokeless tobacco products.

The non-user subjects had a mixture of positive and negative gram-stained bacteria in their mouths, further providing data proving that the vapor disrupts a healthy oral microbiome (Figure 2). In forensic science, these findings can be used to distinguish between suspects during an investigation since using smokeless tobacco products over time results in a change in overall bacterial composition and diversity (Figure 1). The most common morphology type was small white circles (Figure 3). As found in many studies and taught in classes of Microbiology, lysozyme is essential for our mouth microbiome. It keeps Gram-positive bacteria in check and allows Gram-negative bacteria to take up some space in the mouth. The difference between the smokers' and non-smokers' gram tests may show that there is a chemical element found in smokeless tobacco that eliminates or affects lysozyme enzymes, favoring growth of Gram-positive bacteria.. This finding could help with creating criteria for microbial profiles in forensics. (8) The finding that non-smokers had consistent diversity of morphotypes, while smokers displayed variable levels of diversity, suggests that tobacco use may impact microbial populations. Bacteria from the mouth, nose, and hands don't transfer easily to vaping devices. This affects microbial fingerprinting in forensic science. Vaping devices may not be useful for identifying specific individuals based on microbial evidence alone.

However, using smokeless tobacco products can lead to changes in the oral microbiome, which may ultimately affect microbial diversity and lead to identifying smokers based on their affected microbiome. As demonstrated in this study, the standard deviation of microbial diversity between smokers and non-smokers was highly variable, with smokers displaying a wide range of mean values and levels of diversity. (Table 1) These findings suggest that microbial fingerprinting as a tool to identify smokers may be complicated by the potential impact of tobacco use on microbial populations. Other attributes such as gender, lifestyle, food habits, age, consumption history, health status, and socio-economic conditions of the participants can significantly alter the oral microbiome, which would change final results (4). This study would benefit greatly with more research that used these listed attributes in its final data

Figure 1.
Mean and Standard Deviation of bacteria found in Smokers and Non-Smokers mouths.



collection. The individual Smokeless tobacco products are known to hold microbes such as bacteria and fungi. (3) Our findings in ELF BAR products tested show both bacteria and fungi at different levels, which differ depending on the user or users. Further research is needed to understand the potential harm of ELF BAR products on the user's oral microbiome and health and to accurately identify individuals based on varying levels of bacteria and fungi found in testing.

LITERATURE CITED

National Academies of Sciences, Engineering, and Medicine; Health and Medicine Division; Board on Population Health and Public Health Practice; Committee on the Review of the Health Effects of Electronic Nicotine Delivery Systems. Public Health Consequences of E-Cigarettes. *National Academies Press* 2018

Smyth, E.M., Kulkarni, P., Claye, E., Stanfill, S., Tyx, R., Maddox, C., Mongodin, E.F., & Sapkota, A.R. Smokeless tobacco products harbor diverse bacterial microbiota that differ across products and brands. *Appl Microbiol Biotechnol* 101, 5391–5403 (2017)

Speruda M, Piecuch A, Borzęcka J, Kadej M, Ogórek R. Microbial Traces and Their Role in Forensic Science. *Journal of Applied Microbiology*, vol. 132, no. 4, 2547–2557. (2022)

Rivera, A.J., Tyx, R.E. Microbiology of the American Smokeless Tobacco. *Appl Microbiol Biotechnol* 105, 4843–4853 (2021)

Liu, M., Jin, J., Pan, H., Feng, J., Cerniglia, C.E., Yang, M. & Chen, H. Effect of Smokeless Tobacco Products on Human Oral Bacteria Growth and Viability. *ScienceDirect* 42, 152-161

Vishwakarma, A., Verma, D. Microorganisms: crucial players of smokeless tobacco for several health attributes. *Appl Microbiol Biotechnol* 105, 6123–6132 (2021)

Lungile , Nkosi. "20-Year Trends in Tobacco Sales and Self-Reported Tobacco Use in the United States, 2000–2020." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 28 July 2022

Sintija, M., Rostoka, D., Skadiņš, I., Reinis, A., Priedīte,V., Koka, R., Lauva, D. & Kroiča, J. The Oral Microbiome of Smokeless Tobacco Users in Latvia. Proceedings of the Latvian Academy of Sciences 71, 33-37 (2017)Microbiology, vol. 132, no. 4, 2547–2557. (2022)