

# THE ORAL MICROBIOME OF SMOKELESS TOBACCO USERS IN LATVIA

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*Snus is a tobacco product containing nicotine and is widely used in Sweden. Now it is becoming more and more popular among young athletes and teenagers in Latvia, even though it is forbidden for sale in the European Union. The use of snus is considered to induce various oral illnesses, especially periodontal diseases, diabetes, heart and cardiovascular diseases as well as cancer. Comparison of the microbiome of saliva and tooth biofilm in snus tobacco users with that in people who never use snus showed that, the number and diversity of periodontal pathogenic microorganisms was much higher in samples taken from snus users. The observed features of the oral microbiome, such as the presence of periodontal pathogens and their high concentration, may have adverse effect on periodontal tissues of snus users and their general health in the future.*

**Key words:** *microbiome, biofilm, periodontal pathogens, snus.*

## INTRODUCTION

Snus is a tobacco product, which is used by placing it under the upper or lower lip. The nicotine present in this product is absorbed in the body through capillaries in the mucous membrane. Sale of snus is prohibited in the European Union, except for Sweden. The European Union introduced this ban in 1987. Although Sweden joined the European Union in 1995, this ban is not in force, based on historical traditions of using snus in Sweden (Peeters, 2013). The use of snus is increasing in Sweden, which is likely due to the numerous restrictions on smoking in public places. When using snus, nicotine from the tobacco does harm only the user himself, fellow citizens are not threatened, in contrast to danger from passively inhaled smoke from cigarettes. There is a wide variety of snus available in the market, ranging from flavoured snus (vanilla, peppermint, strawberries, etc.) intended mainly for women, up to very strongly flavoured snus, for example, enriched with xylitol and a taste of smoke, which for the first-time users can cause not only headache but also nausea. Snus is industrially manufactured and sold in bulk (loose snus) or in small sachets (portion-packed snus) with an average weight of 0.9 grams. Studies have shown that a snus user on average uses 11 grams of snus per day if it is packed in sachets, or 29–32 grams if packed in bulk form. Usually the duration of usage of one serving is 60–70 minutes (Digard *et al.*, 2009). According to theoretical calculations, about 12.3%, or 1 178 628 people are using snus in Sweden. In 2016, the population of Sweden was 9 582 339, of which 20.7% were male

snus users and 3.5% were female snus smokers (Leon *et al.*, 2015).

In recent years, the use of snus is becoming increasingly popular also in Latvia. Although there is no statistical data concerning the number of snus users in Latvia, there is information that the number of users of this tobacco product is increasing. It is more often used by athletes, in particular by young hockey and floorball players. Snus can be legally imported into the country if the importer carries the product for his own personal use. Infringements arise when snus is imported for sale. There is an illegal chain of distributors currently in Latvia. In addition, the cheaper snus *Odens*, which is also one of the strongest smokeless tobacco products, is most widely used in the Latvian market (*Odens*, Sweden), but there are also other types of snus, such as *Thunder*, *General* (both *Swedish Match*, Sweden) available in the market. It is wrong to believe that snus is not used because it is banned.

Several studies carried out in Sweden have shown how different ingredients of snus can harm the users' health. They show the detrimental effects of smoking and using snus on the general condition of the body, as well as describe the characteristics of snus produced by different producing countries, and how the use of snus correlates with the alcohol dependence (Norberg *et al.*, 2015). No studies on the harm of snus have been carried out in Latvia. Long-term use of snus can induce different changes in the microbiota of biofilm in the oral cavity, caused by changes in the

microbiome, such as gingivitis or periodontitis (Fisher, 2005). The use of snus increases the risk of atherosclerosis and other heart and cardiovascular diseases (Nakano *et al.*, 2009), as well as it might induce pancreatic cancer (Boffeta *et al.*, 2008). The aim of this study was to determine the microbiological characteristics of supragingival and subgingival biofilms in snus users.

## MATERIAL AND METHODS

Survey of snus users and a control group consisting of non-users of tobacco products was carried out. The questionnaire of the survey included questions about age, occupation, daily oral hygiene habits, general health condition, illnesses, frequency of tobacco product use, etc. Description of the oral microbiome was carried out on 20 patients: 10 snus users and 10 were non-smokers (control group). The study and control groups consisted of young and healthy men, working or studying, often playing sports, mostly hockey or floorball players, with average age of 22 years. The most popular types of snus used in the snus user group were *Odens* (*Odens*, Sweden), *Goteburgs Rape* and *Thunder* (*Swedish Match*, Sweden).

Native preparations of biofilms were examined, using a phase-contrast microscope (*Iponacology*, Japan), to determine the proportional relationship between the qualitative and quantitative composition of the oral microbiome and microorganisms.

Saliva of patients was placed in a sterile container and transported to the laboratory of Riga Stradiņš University department of biology and microbiology, and incubated in TSB (Trypticase Soy broth, Oxoid, UK) medium at 37° C for 48 hours. The saliva preparations were stained by Giemsa stain procedure and the morphological characteristics of the microorganisms were evaluated microscopically (Nikon, Japan).

For qualitative and quantitative examination of anaerobic microbiota, gingival sulcus biofilms from the supragingival and subgingival area were collected in a sterile Eppendorph tube. The quantity of bacteria was estimated by real time polymerase chain reaction (RT-PCR Parodontocreen, DNA-Technology). The quantity of bacteria was expressed by the reference interval Lg/equivalents/sample. The presence of periodontal pathogenic bacteria *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia* was determined in the control group and the users.

Significant differences were determined by the Mann-Whitney U test. The authorisation No. 22/28.01.2016 to carry out the study was issued by the Ethics Committee of Riga Stradiņš University.

## RESULTS

Questionnaire answers showed that 6 of 10 non-smoking tobacco group patients used snus tobacco throughout the day nonstop (more than eight hours a day), 2 of 10 use it a few

times a day — about four hours a day and 2 of 10 use it a few times a week. The most popular snus brand was *Odens Cold Extremely White Portion* snus (Sweden). One sachet of this snus contains 22 mg nicotine (Table 1). Non-smoking tobacco users that use snus nonstop or replace a sachet every 30–60 minutes equates with intake of more than 154 mg nicotine daily (two packages of snus every week).

Using the RT-PCR Parodontoscreen method, pathogenic microorganisms were not identified in the oral cavity of most of the patients in the control group. A small amount of periodontal pathogenic bacteria (bacterial concentrations of *Tannerella forsythia* < 10<sup>3</sup> and *Treponema denticola* < 10<sup>2</sup>) was detected in samples taken from three control group patients, which can be treated by using local antimicrobial agents (Table 2). These bacterial results among the control group patients can be explained by insufficient oral hygiene habits, as some patients indicated in the questionnaire that they did not use dental floss and brushed their teeth less than once a day. In the snus users' group, the presence of pathogenic periodontal microorganisms was detected in material taken from all patients. Bacterial concentrations of *Aggregatibacter actinomycetemcomitans* (>10<sup>4</sup>), *Tannerella forsythia* (>10<sup>5</sup>), *Treponema denticola* (>10<sup>4</sup>) in periodontal pockets were higher than in the control group. Bacterial concentration of *Porphyromonas gingivalis* (< 10<sup>5</sup>) (*p* = 0.42) was acceptable in periodontal pockets. Presence of *Prevotella intermedia* was negative in both groups (Table 3).

Morphological examination of saliva and biofilm samples under phase-contrast microscopy showed high concentration of protozoa and bacteria. More than a half of the samples taken from snus users contained *Entamoeba spp.* The presence of *Entamoeba spp.* is associated with a significant change in the oral microbiome and, no doubt, the development of an anaerobic biofilm (Bonner, 2014). In this study *Trichomonas spp.* was found in one of the samples taken from the snus users. Morphological examination of samples taken from snus users indicated significant diversity of

Table 1  
CONCENTRATION OF NICOTINE IN DIFFERENT TYPES OF SNUS

Different brands of snus (produced in Sweden)	Nicotine per pouch (per 1g), mg
Genereal Classic Portion snus	8.0
XRANGE general slim large portion snus	8.0
Eitan vit portion snus	8.0
Goteburgs Rape original	8.0
Grov stark portion snus	8.0
Catch white spaermint	8.0
The Lab 02 slim portion strong	12.0
The Lab 06 slim portion strong	20.0
Mustang vit	12.0
Odens White	8.0
Odens Cold Extreme white Portion snus	22.0
WOW. Wintermelon white	8.0
Siberia Brown Extremely Strong portion snus	43.0
Skruf Slim Fresh white portion snus	8.0

Table 2

QUANTITATIVE AND QUALITATIVE COMPOSITION (LG/EQUIV/IN THE SAMPLE) OF THE MICROBIOME IN THE CONTROL GROUP

Patient	<i>A. actinomycetemcomitans</i>	<i>P. gingivalis</i>	<i>P. intermedia</i>	<i>T. forsythia</i>	<i>T. denticola</i>
1	negative	negative	negative	1.50	2.00
2	negative	negative	negative	1.50	2.00
3	negative	negative	negative	3.90	negative
4	negative	negative	negative	negative	negative
5	negative	negative	negative	negative	negative
6	negative	negative	negative	negative	negative
7	negative	negative	negative	negative	negative
8	negative	negative	negative	negative	negative
9	negative	negative	negative	negative	negative
10	negative	negative	negative	negative	negative
Reference range	< 4.0	< 5.00	< 4.50	< 5.00	< 3.50

Table 3

QUANTITATIVE AND QUALITATIVE COMPOSITION (LG/EQUIV/IN THE SAMPLE) OF THE MICROBIOME IN THE SNUS USERS' GROUP

Patient	<i>A. actinomycetemcomitans</i>	<i>P. gingivalis</i>	<i>P. intermedia</i>	<i>T. forsythia</i>	<i>T. denticola</i>
1	negative	negative	negative	2.10	1.40
2	4.4↑	1.80	negative	1.40	1.90
3	negative	negative	negative	5.40↑	4.90↑
4	negative	1.60	negative	2.30	2.10
5	negative	negative	negative	2.10	1.40
6	negative	negative	negative	1.50	4.90↑
7	negative	negative	negative	1.60	2.10
8	negative	1.20	negative	5.30↑	2.10
9	4.10↑	negative	negative	2.20	1.50
10	negative	negative	negative	2.60	2.20
Reference range	< 4.0	< 5.00	< 4.50	< 5.00	< 3.50

↑ – exceed reference range

pathogens not typical to the microbiota of the normal oral cavity. Questionnaire results showed that only 70% patients of the target group brushed their teeth twice a day, whereas the others (30%) performed this once a day or less. According to the survey, only 65% of participants included in the study used dental floss, whereas others have never used it in their oral hygiene routine. The results of the study showed that there was no relation ( $p = 0.42$ ) between the oral hygiene habits and the diversity of microorganisms in the oral cavity of snus users, i.e. if snus was used, the better oral hygiene habits would not result in individual improvements, and there would be no therapeutic effect or it would be temporary and non-lasting.

## DISCUSSION

The average age of smokeless tobacco users in this study was 22 years. A study in Sweden, performed over a period of seven years, showed that 15.6% of women and men in the age group of 30–34 years use snus, 11.5% in the age group of 35–44 years, 17.3% in the age group of 45–55 years and 15.9% in the age group of 55–62 years (Åkesson *et al.*, 2016). We can conclude that smokeless tobacco is more popular among young male adults, as all volunteers were males. The results indicate that smokeless tobacco users will have higher risk of periodontal diseases due to high *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* concentration in periodontal pockets. Even a low amount of *Porphyromonas gingivalis* is associated with severe periodontitis (Morita and Wang, 2001). Use of snus affects not only the oral cavity but also other organs of the body. For example, in a study with rats to determine the impact of snus on different organs of the body it was concluded that a toxicity was higher in the oesophagus, liver, kidneys, and lungs. It should be also noted that rat weight was lower in group with high use of snus (Yu *et al.*, 2016).

Over 50% of Sudanese men have suffered from oral cancer (Erikson *et al.*, 2015). Although smokeless tobacco increases risk of oral cancer development it is not associated with oral caries (Åkesson *et al.*, 2016). As the questionnaire showed that almost half of participants had poor oral hygiene and had evidence of periodontal disease, there is also higher risk of pancreatic cancer (Huang *et al.*, 2016). In order to prevent the presence of microorganisms in the oral cavity either local antibiotics intended for low concentration of microorganisms, or general antibiotics intended for increased concentration of microorganisms, should be used (Anonymous, 2004). Some snus products are enriched with menthol to make it more attractive to users. However, in a study, where tobacco products with menthol were compared to general tobacco products, it was discovered, that patients preferring menthol-enriched products, suffered more often from depression and anxiety than those who used tobacco products of different tastes (Cohn *et al.*, 2016).

Latvia does not have a long-term history of using snus, therefore people are not yet aware of the possible health risks of this type of tobacco product. In Latvia, smokeless tobacco is used mainly by men, whereas women do not like it because of its distinctive taste and smell. Latvians have very little knowledge on the use of snus, its effects and damage to the health. Some snus users consider this kind of product to be harmless. Therefore, Latvians have insufficient knowledge about snus. Some users do not know that smokeless tobacco products should be stored in a refrigerator. There are numerous studies on change in nitrate concentration in snus. For example, if a pouch containing snus is stored for four weeks at a temperature of +37 °C, the level of N-nitrates increases significantly (Djordjevic *et al.*, 1993). In Sweden snus is stored in refrigerators at a temperature of +4 °C to keep it fresh. Snus users in Latvia do not

follow these storage rules, likely causing increase of nitrate concentration in these smokeless tobacco products.

There are different effects of processing smokeless tobacco products on levels of Tobacco Specific Nitrosamines (TSNAs). Nitrates are considered to be carcinogens and may cause malignant tumours. Pasteurised Swedish snus contains 601–5850 ng/g TSNAs; snus that is fermented in a factory (USA) contains 1520–20500 ng/g TSNAs, while snus that is fermented by composting (Sudan) contains 295 000–992 000 ng/g TSNAs (Eriksen *et al.*, 2015). Questionnaires showed that some users have high nicotine intake in their body. Furthermore, snus pH affects the level of available nicotine: in snus with pH 5.84, 1% of the nicotine is in a form free for absorption, whereas in snus with pH 7.99, 59% of the nicotine is in a free form (Brunnemann *et al.*, 2002). Therefore, it can be concluded that pH affects absorption of nicotine: the higher the pH value, the more nicotine is absorbed. There are products similar to snus in other regions of the world, for example, *naswar* (*насва́р*, in Russian) is widely used in Russia and in Asian countries such as Uzbekistan, Kyrgyzstan, and Azerbaijan (Leon *et al.*, 2016). *Toombak* is generally used in Sudan and Iraq. *Toombak* contains 302000–992000 ng/g TSNAs, and *naswar* contains 478–1380 ng/g TSNAs (Siddiqi *et al.*, 2015). *Toombak* users also consider it to be stronger. The ingredients of American snus are different from Swedish snus, as American smokeless tobacco contains higher levels of tobacco specific nitrates and carcinogens (Rodu and Jansson, 2004). As both *toombak* and *naswar* contain higher levels of nitrates, it can be concluded that these types of snus products have a more negative effect on the microbiome of the oral cavity and the general health condition than the smokeless tobacco products from Sweden. Swedish snus is refined and Sweden follows strict quality standards for this product, such as the Gothiatek quality standard, which is the main quality standard for *SwedishMatch* snus producers.

A recent study on snus *Afzal* from Oman showed that it exceeded the limits for *N*-Nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in tobacco products (Al-Mukhaini *et al.*, 2016). The smokeless tobacco products produced in Russia, Sudan, and other Asian countries bear no information on compliance with the quality standards of those countries and the quality of refinement, which suggests that snus produced in these countries may contain other admixtures and have greater effect on oral health.

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## MUTES MIKROBIOMA ĪPATNĪBAS BEZDŪMU TABAKAS LIETOTĀJIEM LATVIJĀ

Bezdzūmu tabaka ir nikotīnu saturošs tabakas produkts, plaši lietots Zviedrijā, kas kļūst arvien populārāks jaunu sportistu un jauniešu vidū Latvijā, neraugoties uz to, ka tā pārdošana Eiropas Savienībā ir aizliegta. Bezdzūmu tabakas lietošana ir saistīta ar dažādām saslimšanām mutes dobumā, it īpaši periodonta slimībām, kā arī ar cukura diabētu, sirds un asinsvadu slimībām, onkoloģiskajām slimībām. Salīdzinot bezdzūmu tabakas lietotāju siekalu un zobu biofilmu mikrobiomu ar tabakas nelietotāju mikrobiomu, konstatēts, ka bezdzūmu tabakas lietotāju paraugos bija daudz lielāks patogēno periodonta mikroorganismu skaits un lielāka sugu daudzveidība. Pētījumā noskaidrotās mutes mikrobioma izmaiņas — periodonta patogēno baktēriju klātbūtne, to augstā koncentrācija var apdraudēt bezdzūmu tabakas lietotāju periodonta audus, kā arī vispārējo veselību nākotnē.