IMPACT OF TOBACCO SMOKING
ON ORAL MICROBIOTA –
A CASE-CONTROL STUDY

Abstract. Impact of tobacco smoking on oral microbiota – a case-control study. Abdulrahman Ali Hattan, Essa Ali Hattan, Abdulaziz Maree Alqahtani, Omar Saud Alqutaym, Refdan Obeid Alqahtani, Khaled Ghormallah Alzahrani, Abdulrahman Abdullah Al-Otaibi, Omar Mufi Aldwsari, Khalid Mansour Alkhathlan, Mohammed Abdullah Aldossari. Oral microbiota is a vital part of human microbiota, including bacterial, protozoan, viral and fungal species. Beneficial microbes form biofilms to form a first-line defense against harmful microorganisms. Tobacco smoking is considered a major environmental factor affecting the orodental microbiota. Smokers harbor more pathogenic microbes than non-smokers. In fact, cigarette smoking exposes the oral cavity to a large number of toxicants, perturbing the oral microbial ecology through various mechanisms. In Saudi Arabia, research on the impact of tobacco smoking on oral microbiota is still lacking. Therefore, this case-control study is an important addition to the literature in terms of tobacco use and its effects on oral microbiota and oral hygiene. 130 men were recruited for this study, including 65 smokers and 65 non-smokers. The following parameters were recorded for all 130 participants – age, weight, height and education. The aim of this study was to investigate and compare the effect of tobacco smoking on the oral microbiome of smokers and non-smokers. The majority of the smokers were young adults between the ages of 21 and 30 inclusive (n=27). The results show that excessive microorganism growth was seen in smokers to a greater degree than non-smokers (38.5% of smokers vs. 8.8% of non-smokers). Not surprisingly, a significant majority (85.3%) of non-smokers had moderate microorganism growth compared to only 53.8% of smokers. Cigarette smoking facilitates excessive growth of oral microorganisms, predisposing smokers to various periodontal diseases. In fact, smoking perturbs the balance of oral microbiota, producing a viable environment for microbes to cause diseases. Further large scale prospective studies are required to determine the exact mechanism that causes tobacco to affect oral microbiota.
Oral microbiota refers to several hundreds of diverse species of microbes living in the oral cavity. According to one estimate, we have more cells that are prokaryotic in our body than those of eukaryotic cells [1]. In fact, every tenth cell in our body is human in nature. Oral microbiota is a vital part of human microbiota, including bacterial, protozoa, viral and fungal species. This diverse ecological community of microbes may be associated with human oral mucosa in the form of commensalism, parasitism and local or opportunistic pathogens. Commensal microbiota are beneficial to human health and wellness. Normal oral microbiota do not cause diseases, and also keep other organisms away from mucosal surfaces, as they prevent their adherence to these surfaces. In this context, beneficial microbes form biofilms that form a first-line defense against harmful microorganisms by preventing their attachment to the mucosal surfaces. In addition, these biofilms degrade toxins, contribute to the maturation process of the immune system, synthesize vitamins, and assist digestion. In fact, oral microbiota and human beings coevolved over thousands of years; but this relationship has been significantly affected by changes in societal norms and the environment [2]. Therefore, the majority of oral microbiota cause a number of diseases in human beings e.g. tonsillitis, osteomyelitis, cardiovascular diseases, aspiration pneumonia, dental caries and periodontal disease [3, 4].

Fortunately, the majority of human microbial flora is beneficial to the human body, protecting it from several harmful conditions. However, some of the microbes transit from a commensal relationship with the human body to pathogenicity, due to reasons which are poorly recognized. One of the reasons may be lifestyle changes that adversely affect the commensal and symbiotic relationships of oral microbes and the host [5]. The most important lifestyle factors that affect oral microbiome include diet, the industrial revolution, and the use of antibiotics [4]. For example, a carbohydrate-rich diet significantly affects oral microbiota. The proposed mechanism is that a carbohydrate-rich diet offers readily available dietary sugars for microorganisms. In fact, dietary sugars are a favourable medium for acidogenic and aciduric bacteria that cause orodental disease [6]. Thus, the transition from natural to unnatural or oral microbiota dysbiosis leads to pathogenicity, causing oral as well as systemic diseases [7].

Tobacco smoking is considered a major environmental factor affecting the orodental microbiota. Smokers harbor more pathogenic microbes than non-smokers. In fact, cigarette smoking exposes the oral cavity to a large number of toxicants, perturbing the oral microbial ecology through various mechanisms e.g. oxygen deprivation and antibiotic effects [8]. This dysbiosis leads to several pathogenic conditions. Studies have revealed that 42% of periodontitis in the United States is caused by tobacco use [9]. Additionally, tobacco use is associated with a severe and extensive form of orodental disease, a significant health issue worldwide. In this context, bacterial agents play a major role in the development of periodontal disease. Moreover, cessation of tobacco smoking results in an alteration in the microbial recolonization.

Kumar et al. [10] studied the effect of cigarette smoking on the composition and proinflammatory characteristics of the biofilm in a de novo plaque formation, including 15 smokers and 15 non-smokers (who had never smoked). They reported that smokers demonstrated more diverse and unstable bacterial colonizations in marginal as well as subgingival biofilms, as compared those individuals who were non-smokers. Additionally, the smokers showed early proinflammatory response to the colonization. This shows that cigarette smoking alters the microbiota and creates an environment favourable for pathogenic bacteria, leading to a number of periodontal conditions. In Jeddah KSA, Baljoon et al. [11] studied the effect of water pipe smoking on the periodontal bone height of 355 individuals. They reported that water pipe smoking reduces periodontal bone height in a similar manner.
to those who smoke cigarettes. Thus, tobacco smoking exposes individuals to harmful microbiome, causing periodontal disease. In a similar fashion, tobacco smoking exacerbates the role of microorganisms other than bacteria, leading to the conditions associated with those microorganisms. For example, a strong association has been observed between tobacco use and exposure to infection with the human papilloma virus type 16 (HPV16) [12]. This means that tobacco smoking increases the chances of exposure to HPV16, a carcinogenic virus.

Elaboration of more and more environmental factors affecting the oral microbiota will help improve strategies regarding oral hygiene, treatment trends and preventive measures. In Saudi Arabia, research on the impact of tobacco smoking on oral microbiota is still lacking. Therefore, this case-control study is an important addition to the literature in terms of tobacco use and its effects on oral microbiota and oral hygiene.

**MATERIALS AND METHODS**

This case-control study was conducted at Prince Sattam University Hospital. 130 men were recruited for this study, 65 of whom were smokers and 65 non-smokers. All participants signed a valid informed consent form prior to inclusion in this study.

The selection criteria for the smoking group were as follows: Male gender, human immunodeficiency virus (HIV) and diabetes mellitus free, and a smoking duration of at least 6 months. Patients who were treated with antibiotics in the 30 days prior to the sample collection were excluded from the study.

The selection criteria for the non-smoking group were as follows: Male gender, HIV and diabetes mellitus free, and a history of non-smoking. As with the smoking group, patients treated with antibiotics in the 30 days prior to sample collection were excluded from the study.

The following parameters were recorded for all 130 participants: age, weight, height and education. The aim of this study was to investigate and compare the effect of tobacco smoking on the oral microbiome in smokers and non-smokers.

For the analysis of oral cavity micro-flora, samples were collected by an expert physician using standard protocols (X1). After sampling, the cotton swabs were immediately inserted into the transport media (which were purchased from Deltalab, S.L., Spain). Duplicate sampling was also done by using conventionally prepared sterile swabs, which were transferred onto sterile cotton-plugged test tubes containing saline at pH 6.8/360°C. Swabs were sent to the laboratory for culture and for identification.

The samples were plated in different media for the evaluation of possible bacterial and fungal members. Different microbiological growth media, such as Nutrient agar, Nutrient broth, Sheep Blood agar plates, Mannitol Salt agar, Eosine Methylene Blue agar, Muller-Hinton agar and MacConkey’s agar were used to detect the bacterial flora by incubating for 36 to 48 hours at 370°C, as per standard methods. Sabouraud Dextrose agar (SDA) and Potato Dextrose agar (PDA) were used for the primary elucidation of possible fungal members from the samples by incubating at 300°C for four to six days, in accordance with standardized protocols (X2, X3). The media were purchased from different international manufacturers, such as OXoid Ltd., England, Scharlau, Scharlub S.L., Spain, SMPL (Saudi Prepared Media Ltd., Company) K.S.A., etc.

The growth morphology was analysed, and selected colonies were subjected to basic standardized staining procedures, such as Gram’s staining, Giemsa’s staining, Lactophenol Cotton Blue (LCB) mount, KOH mount, etc. (X4). The bacterial isolates were subjected to primary screening biochemical tests, such as Catalase, Oxidase, IMViC, etc. The stains and chemicals/reagents used were obtained from Avonchem, U.K., Crescent Diagnostics, K.S.A., LobaChem, India, etc. To determine the microorganism growth rate we used the spectrophotometer method. The amount of light absorbed by the bacterial culture was measured. To measure bacterial concentration, a wavelength of 600 nm (A600) was used. We carried out the harvesting of a culture in the period of the early-log phase of cell growth. When we measured the growth rate of bacteria in the culture, an OD of 0.5-.7 indicated that the bacteria were in the early to mid-log phase of their growth.

All tests were performed in triplicate and the results were recorded.

**RESULTS AND DISCUSSION**

The specific demographic details of the 130 participants recruited for this study can be seen in Table. The majority of the participants were adolescents and young adults between the ages of 11 to 30 (n=82). The majority of the smokers were young adults between the ages of 21 to 30 (n=27). 26 out of 130 participants had bad breath, and 92.3% of them were smokers. A test of independence between bad breath and smoking was conducted by way of the Pearson Chi-Square test, which showed a significance of χ²(1,130)=21.202, P<0.001. This suggests an association between smoking and bad breath, in that smokers are more likely to have bad breath compared to non-smokers.

27 out of 130 participants exercised regularly, and 22.7% of them were smokers. A test of independence between bad breath and smoking was
conducted by way of the Pearson Chi-Square test which showed a significance of \( -\chi^2 (1,130)=11.967, P=0.0005415. \) This suggests an association between smoking and exercise, in that smokers are less likely to exercise regularly compared to non-smokers.

45.9% of university graduates and 50% of high-school graduates were smokers. However, 90% of participants who were not educated were smokers. A test of independence between education and smoking was conducted by way of the Pearson Chi-Square test which showed a significance of \( -\chi^2 (2,130)=7.0531, P=0.02941. \) This suggests an association between smoking and education, in that smokers are more likely to be less educated compared to non-smokers.

### Demographics breakdown of participants

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Smoking Status</th>
<th>Total</th>
<th>Pearson Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non-smoker</td>
<td>smoker</td>
<td>value</td>
</tr>
<tr>
<td>Education</td>
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<td>53</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>High School</td>
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</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Exercise</td>
<td>Don’t Exercise</td>
<td>43</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1 – 3 days/week</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>&gt;3 days/Week</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>Halitosis</td>
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</tr>
<tr>
<td></td>
<td>No</td>
<td>63</td>
<td>41</td>
</tr>
<tr>
<td>Age group</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>11-20</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>21-30</td>
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<td>27</td>
</tr>
<tr>
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<td>31-40</td>
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<tr>
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<td>51-60</td>
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<td>4</td>
</tr>
<tr>
<td>More than 60</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Referring to Figure 1 and 2, the microbial analysis of the oral swabs obtained from the 130 participants can be evaluated. The prevalence of seven pathogens (five bacterial and two fungal) were analysed for the smoker group and non-smoker group. A comparison of this prevalence provided the basis for us to define three discrete categories – mild growth, moderate growth and excessive growth. We compared the proportion of smokers and non-smokers in each of these three categories, as shown in Figure 3.

Mild growth was defined as a lower number of bacterial colony-forming units (cfus) obtained as a result of sampling (at a particular site of the body) and culture (using standard procedures), compared with the normal / standard / expected value. The change in the quantity could be a result of any of the factors/parameters that brought an alteration in the typical condition as a result of the experiment / procedure under investigation.
Moderate growth was defined as a normal or average number of bacterial colony-forming units (cfus) obtained as a result of sampling (at a particular site of the body) and culture (using standard procedures), as per the established standards and literature references (almost) like the normal / standard / expected value. The lack of change in the quantity of the cfus may suggest that the altered / changed factor / parameter may not have any appreciable influence on the bacterial growth in the reported study / experiment / investigation.

Excessive growth was defined as a higher number of bacterial colony-forming units (cfus) obtained as a result of sampling (at a particular site of the body) and culture (using standard procedures), as per the established standards and literature references, compared with the normal / standard / expected value. The increase in the quantity of the cfus may suggest that the altered / changed factor / parameter may have a favourable effect on the bacterial growth in the reported study / experiment / investigation.
Fig. 3. Comparison Of Microorganism growth Between Smokers and Non-Smokers

The results show that excessive microorganism growth was seen in smokers to a greater degree than non-smokers (38.5% of smokers vs. 8.8% of non-smokers). Not surprisingly, a significant majority (85.3%) of non-smokers had moderate microorganism growth compared to only 53.8% of smokers.

Cigarette smoking significantly perturbs the oral microbiota via various possible mechanisms, e.g. oxygen deprivation and impaired normal host immunity. The present case-control study reported that excessive microorganism growth was seen in the tobacco smoking group to a greater degree than in the non-smoking group. The reason for excessive microbial growth in tobacco smokers may be attributed to the altered oral microbial parameters, which had a favourable effect on the bacterial growth reported in the present study. However, in contrast, a significant majority of non-smokers showed moderate microorganism growth, as compared to the tobacco smokers. Additionally, the present study reported halitosis and lack of exercise in a significant majority of tobacco smokers, as compared to non-smokers. Similarly, 90% of non-educated participants were tobacco smokers. Hence, the present study strengthens the point that health education is a vital step in order to prevent diseases.

In fact, tobacco smoking favours bacterial adhesion to oral mucosal surfaces, increasing the risk of enhanced bacterial growth and subsequent development of diseases, e.g. gingivitis and adult periodontitis [13]. Antolin [14] studied the comparison of bacterial growth in oral cavities of tobacco smokers or chewers and non-tobacco users. The study measured the growth of culture of oral bacteria using a spectrophotometer at 12, 24 and 36 hours of incubation. It reported significant bacterial growth after incubation of 12 hours for tobacco smokers and chewers, as compared to non-tobacco users. On the other hand, the study found no significant difference in bacterial growth between the two groups after 24 hours and 36 hours of incubation. The reason there were no significant differences at 24 and 36 hours was not identified. Wu et al. [7] conducted a large study comprising 1204 adults in the United States, and performed a meta-analysis in order to assess the oral microbiome composition in tobacco smokers and non-smokers. They collected oral wash samples, performed 16S rRNA gene sequencing, and reported that tobacco smoking alters oral microbiome, shifting functional pathways and favouring smoking-related diseases. They reported the depletion of Proteobacteria, and increased growth of Firmicutes and Actinobacteria among tobacco smokers, as compared to non-smokers. This indicates that tobacco smoking perturbs the oral microbiota, disturbing the balance of oral flora and favouring the environment for smoking-related oral and other systemic diseases. Additionally, they reported a similar composition of oral microbiome among former smokers and those who had never smoked. This means that smoking-related changes in oral microbiota are not permanent and revert after smoking cessation [15]. Similarly, Kato et al. [2] also reported that cigarette smoking alters the equilibrium of oral microbiota.
Borningen et al. [16] conducted a case-control study that included 121 oral cancer patients and 242 controls, and compared composition, diversity and function of oral microbiota. They reported significant changes in the composition and function of oral microbiota in participants with oral cancer, tobacco smoking and poor oral hygiene. They identified changes in 24 clades and 12 metabolic pathways in smokers, as compared to non-smokers, demonstrating that smoking lowers alpha-diversity and increases beta-diversity. Additionally, they reported significant changes in microbial functional modules among smokers, e.g. enhanced sugar and phosphate uptake, abundance of metal transport systems, and the GABA gamma aminobutyrate shunt. All of these functional changes support the results of the present study in the way that tobacco smoking alters oral microbiota, which increases the chances of periodontal diseases. Wu et al. [7] demonstrated the same functional changes in their study. Other studies have reported inhibitory effects of cigarette smoking on certain bacterial species. In early vitro studies, tobacco smokers exhibited decreased Neisseria species on their mucosal surfaces [17, 18].

Cigarette smoking increases the mucus and phlegm in the throat, adding to halitosis [19]. In the present study, increased halitosis among cigarette smokers indicates increased bacterial growth in the oral cavity. Hence, the present study again indicates the excessive growth of oral microbiota among tobacco smokers, especially gram-negative bacteria [20]. Similarly, the present study revealed a lack of exercise among cigarette smokers. In this regard, Fukuba et al. [21] reported that cigarette smoking reduces aerobic and non-aerobic power due to problems with muscle contraction activities among smokers.

The strength of the present study is that it clearly documents the excessive growth of oral microbiota among smokers, which increases the risk of diseases. As with every study design, this study also had some limitations. Being a case-control study, selection and observation biases might have altered the outcome.

CONCLUSION

Cigarette smoking facilitates excessive growth of oral microorganisms, predisposing smokers to various periodontal diseases. In fact, smoking perturbs the balance of oral microbiota, producing a viable environment for microbes to cause diseases. Further large-scale prospective studies are required to determine the exact mechanism of tobacco that affects oral microbiota, in order to validate the results of this study.

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