European Journal of Oral Sciences

Eur J Oral Sci 2013; 121: 132–135
DOI: 10.1111/eos.12030
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European Journal of Oral Sciences

Short communication

Characterization of the oral fungal microbiota in smokers and non-smokers

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Monteiro-da-Silva F, Sampaio-Maia B, Pereira ML, Araujo R. Characterization of the oral fungal microbiota in smokers and non-smokers.
Eur J Oral Sci 2013; 121: 132–135. © 2013 Eur J Oral Sci

This study aimed to assess the effect of smoking on the biodiversity of the oral fungal microbiota of healthy young subjects, using an improved culture method that assesses both total and pathogenic viable fungi. Forty individuals (20 smokers and 20 non-smokers) were selected. All individuals presented fungal growth (100% for molds and 92.5% for yeasts), a prevalence higher than previously reported. The most commonly occurring molds were *Penicillium* sp., *Aspergillus* sp., and *Cladosporium* sp. Smokers presented significantly higher levels of yeasts and pathogenic molds than did non-smokers. No differences in fungal prevalence and diversity were observed in smokers and non-smokers following a 30-wk observation period. In conclusion, tobacco smoking may alter the oral mycobiota and facilitate colonization of the oral cavity with yeasts and pathogenic molds. The effect of chronic fungal colonization on the oral health of tobacco smokers cannot be neglected.

Key words: diversity; molds; mycobiome; smoking habits; yeasts

Accepted for publication January 2013

The oral microbiota constitutes one of the most complex communities in the human body and is composed of up to 19,000 phylotypes (1–3). However, this microbiome is continuously exposed to a variety of hostile factors that can affect the microbial balance of the oral mucosa. One of the most relevant factors in this regard is smoking habit, which is quite common in the global world society and is associated with relevant oral diseases, such as periodontitis and oral cancer. The higher prevalence and greater severity of oral disease in smokers might be explained by the altered host immune response as disturbances in immunoglobulin and cytokine levels, altered lymphocyte counts, and impairment of oral neutrophil function demonstrated in smokers (4–6). Numerous studies have reported that in comparison with non-smokers, smokers present enhanced levels of certain bacterial species in supragingival and subgingival plaque, namely members of the orange and red bacterial complexes, including *Bacteroides forsythus, Eubacterium nodatum, Fusobacterium nucleatum* ssp. *vincentii, Peptostreptococcus micros, Prevotella intermedia, Prevotella nigrescens, Porphyromonas gingivalis,* and *Treponema denticola* (4, 7). In contrast, other studies have found no significant differences in the composition of the subgingival periodontopathogenic microbiota of smokers with periodontal disease (5, 8).

With respect to fungi, tobacco smokers have a higher rate of oral *Candida* carriage than do non-smokers (9, 10). A number of studies over the past two decades have found evidence that smoking, either alone or in combination with other factors (such as denture wearing and diabetes), appears to be an important predisposing factor for oral candidosis (11). Furthermore, clinicians suggest that some yeast infections invariably disappear following smoking cessation, without additional therapy (12).

Ghannoum *et al.* (1) analyzed the fungal component of the oral microbiota by pyrosequencing and described several genera previously neglected in the oral cavity. The purpose of the present investigation was to assess the effect of smoking on the biodiversity of oral fungal microbiota – yeasts and molds – of healthy subjects, using improved culture methods followed by biochemical multitesting and molecular methods for identification.

Material and methods

Forty healthy students from the fifth year of the Masters Degree of the Faculty of Dental Medicine of Porto University were invited to participate voluntarily. The Ethics Committee of same faculty approved the consent form and the research protocol. The medical and dental histories of each subject, as well as information on oral hygiene habits, alcohol consumption habits, and contraceptive drug use, were obtained by interview. Oral clinical examination included assessment of probing depth, gingival

recession, and bleeding on probing at six sites per tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual) for all teeth. Caries prevalence was assessed using the decayed, missing or filled surfaces (DMFS) index, and oral hygiene was assessed using the O'Leary plaque control record (13, 14).

Study participants rinsed their mouth with 10 ml of sterile water for 15 s, and the samples were taken up in 250 ml of Sabouraud glucose agar supplemented with chloramphenicol. The culture medium was divided among 10 Petri dishes and incubated at 25°C or 37°C for 7 d, to monitor the growth of total and pathogenic fungi (15). The number of fungal colonies were registered as colony-forming units per ml (CFU/ml) and the growing fungi were identified based on macroscopic and microscopic morphology (16). API ID 32C (BioMerieux, Hazelwood, MO, USA) was used for yeast identification. All fungi showing unreliable traditional identification were recognized by sequencing 18S and internal transcribed spacer (ITS) regions, employing a previously suggested set of primers (17, 18).

A value of $P < 0.05$ was considered statistically significant. The categorical variables were described using relative frequencies (%), whereas continuous variables were described using mean ± SD. The chi-square test of independence was used to analyze the categorical variables and the Mann–Whitney *U*-test was used for continuous variables. The statistical analyses were performed using SPSS v.17.0 (IBM SPSS Statistics, Armonk, NY, USA).

Results and discussion

The final sample included 26 women (65%) and 14 men (35%), aged between 22 and 34 yr (mean age ± SD = 24.0 ± 2.8 yr). The mean oral hygiene index was 45.1 ± 12.1 and the DMFS index was 7.5 ± 7.3.

The non-smoker group comprised 20% men (mean age ± SD = 23.2 ± 1.8 yr), whereas the smoker group comprised 50% men (mean age ± SD = 24.8 ± 3.4 yr). Smokers had consumed a mean of 8.5 ± 6.0 cigarettes a day for a period of 4.5 ± 4.1 yr. No differences were found between smokers and non-smokers regarding oral hygiene, DMFS, and periodontal indexes (Table S1).

All subjects presented colonization of the oral cavity with fungi. Two sets of samples, incubated at different temperatures, were analyzed: (i) the samples incubated at 25°C showed a prevalence of 100% for molds and a prevalence of ~92% for yeasts in both smokers and non-smokers, and (ii) the samples incubated at 37°C showed a prevalence of molds of ~42% in smokers and in non-smokers, whilst the prevalence of yeasts was 55% in smokers and 35% in non-smokers. For general analysis, samples at 25°C were preferable, given that higher recovery rates are actually more representative of the degree of oral fungal colonization in vivo. An incubation temperature of 37°C was preferable for growth of a few pathogenic fungi, such as *Candida albicans* and *Aspergillus fumigatus*.

Table 1 depicts the comparison of mold and yeast counts obtained in oral samples positive for fungi from smokers and non-smokers. Smokers had significantly higher levels of yeasts in samples incubated at 25°C. At 37°C, significantly higher levels of molds and total fungi were found in smokers.

Table 1

Fungal quantification in smokers and non-smokers (positive samples for fungi)

Culture conditions	Smokers CFUs	Non-smokers CFUs	*P*-value
25°C for 7 d			
Yeasts	60.7 ± 97.8* (18)	5.4 ± 36.7* (19)	**0.037**
Molds	5.4 ± 2.4 (20)	5.6 ± 2.1 (20)	0.507
Total fungi	60.1 ± 92.6* (20)	20.6 ± 35.5* (20)	0.068
37°C for 7 d			
Yeasts	98.4 ± 106.4* (11)	28.3 ± 25.6* (7)	0.258
Molds	1.5 ± 1.7 (8)	0.4 ± 0.2 (9)	**0.019**
Total fungi	73.0 ± 100.1* (15)	14.4 ± 22.6* (14)	**0.041**

Colony-forming unit (CFU) values are given as mean CFUs/ml ± SD with the number of samples in parentheses. *P*-values were calculated using the Mann–Whitney *U*-test.
*High SD values as a result of the presence of 30% of smokers and 10% of non-smokers with yeasts counts >80 CFU/ml. *P*-value in bold represent statistically significant differences.

Based on microscopic features and API results, we were able to identify the following yeasts: *Candida* spp. (*C. albicans*, *Candida parapsilosis*, and *Candida tropicalis*) and *Rhodotorula* sp. Seven different genera of molds were identified: *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Trichoderma*, *Scedosporium*, and *Rhizopus*; ~70% of molds were not identified (Table S2). *Penicillium* sp. molds were those most frequently identified, followed by *Aspergillus* sp. and *Cladosporium* sp. Within the genus *Aspergillus* the most frequent species was *A. fumigatus*, which was found in 40% of all subjects. Other species, such as *Aspergillus flavus* and *Aspergillus glaucus*, were also isolated sporadically. Molecular identification revealed few atypical colonies of *Penicillium* sp. and *Cladosporium* sp. (non-sporulating molds with white branches and septate hyphae). Details on the quantification of each fungal phylotype in smokers and non-smokers are presented in Table S2 as well as individual information on fungal phylotypes (Figure S1).

To assess individual and temporal variation of oral fungi colonization, a follow up of 10 subjects was carried out 6 months after the first sampling (data not shown). The demographic characteristics and smoking habits of these individuals in the follow-up sample were similar to those of the initial sample. The analysis revealed that the participants were colonized with the same fungal taxa at the two sampling periods, regardless of smoking habit. Frequencies of isolation of oral fungi, as well as the concentration of fungi, were constant over the 6-month observation period. Other variables, such as oral contraceptive drugs and alcohol consumption habits, did not influence the prevalence and growth of fungi ($P > 0.05$).

The study aimed to characterize the oral mycobiota and to assess the effect of tobacco smoking on the prevalence and concentration of viable fungi in the oral

cavity of healthy participants showing good indices of oral health and hygiene. All subjects showed colonization of the oral cavity with fungi. However, higher levels of oral colonization with both yeasts and molds were found in tobacco smokers. The oral fungi identified in this study included the yeasts *Candida* sp. and *Rhodotorula* sp. and a variety of mold genera. Our results are consistent with those obtained recently by Ghannoum *et al*. (1). By taking up the oral rinse samples in liquid Sabouraud agar, it was possible to isolate and identify a higher number of yeasts and molds than previously reported using traditional culture methods. The diversity of yeasts and molds found in the present study has never been reported previously in healthy young individuals employing culture methods (19).

We did not find any differences in the prevalence and concentration of oral fungi among smokers and non-smokers. However, the yeast counts, particularly *Candida* sp., seemed somewhat higher in smokers but did not attain statistical significance. *Candida* sp. are considered as the most important and prevalent colonizing fungi of the oral mucosa, being associated with oral diseases, especially in immune-compromised hosts (1, 20). Our results are consistent with the literature in that the rate of oral candida carriage was higher in smokers than in non-smokers (21–23). Some candidal infections invariably disappear following smoking cessation (12).

Furthermore, smokers may present higher counts of pathogenic molds (obtained upon culture at 37°C), suggesting that the growth of these fungi is favored by smoking. In this respect it is of interest that several authors reported a high risk of invasive and chronic pulmonary aspergillosis in tobacco and marijuana consumers (24–26). Although adverse effects related to exposure to molds are rarely reported in healthy individuals, the potential effects of chronic exposure to relatively high concentrations of mold should not be disregarded. Most fungal species found in the oral mucosa, particularly *Penicillium* sp., are mycotoxin producers. Mycotoxins are responsible for well-known toxigenic activities, causing the inhibition of protein synthesis and immunosuppression (27).

The immune-system deficiencies in smokers highlight the importance of oral fungi colonization, mainly with respect to opportunistic pathogens such as *Candida* spp. and *Aspergillus* spp. The oral mucosa could function as portal for systemic fungal infections, particularly in immunocompromised individuals.

It is important to note that the presence of molds in the oral habitat may be a result of transient colonization. However, it is interesting that the colonization profile of both smokers and non-smokers was maintained over a 6-month period.

In conclusion, tobacco smoking may alter the oral mycobiome, facilitating the colonization of the oral cavity and favoring certain yeasts and pathogenic molds. Although the underlying mechanisms are still unclear, possible alterations in the host immune response could be of relevance. Our data highlight the importance of tobacco control because it has been proven that smoking cessation alone may reverse opportunistic fungal infections in the oral cavity and probably also in other body sites.

Acknowledgements: R.A. is supported by Fundação para a Ciência e a Tecnologia (FCT) Ciência 2007 and by the European Social Fund. Nephrology of Research & Development Unit and IPATIMUP (Associate Laboratory) of the Portuguese Ministry of Science, Technology and Higher Education are partially supported by FCT.

Conflicts of interest: The authors report no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Overall distribution of different mold phylotypes in each individual.

Table S1. Clinical characterization of smokers and non-smokers.

Table S2. Fungi genera quantification and prevalence in oral samples from smokers and non-smokers at 25°C and 37°C.