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Interindividual variability and intraindividual stability of oral fungal microbiota over time

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Abstract

Oral microbiota is one of the most complex and diverse microbial communities in the human body. In the present study, we aimed to characterize oral fungi biodiversity and stability over time in a group of healthy participants with good oral health. Oral health and oral fungal microbiota were evaluated in 40 healthy individuals. A follow-up of 10 participants was carried out 28 weeks and 30 weeks after the first sampling. Oral rinse was collected and incubated in a fungal selective medium at 25°C and 37°C for 7 days. Fungi were identified based on macro- and microscopic morphology. API/ID32C was used for yeast identification, and molecular techniques were used to identify the most prevalent nonidentified moulds, mainly by sequencing 18S and internally transcribed spacer regions. Moulds were recovered from all participants and yeast from 92.5%. The most frequently isolated fungi were *Candida* spp., *Rhodotorula* spp., *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* spp. The oral fungal community presented a high interindividual variability, but the frequency and quantification of each fungal taxon was constant over the 30-week observation period, showing a consistent intraindividual stability over time. The intraindividual stability opposed to interindividual variability may suggest a common and a variable group of fungi in the oral cavity.

Key words: fungi, yeast, mould, oral health, mycobiome.

Introduction

The oral microbiome is one of the most complex and diverse microbial communities in the human body [1–3]. The microorganisms living in the oral cavity, as well as their interrelationships, are essential components in the balance between health and disease. Thus, it is crucial to study the microbiology of the human mouth, which is the portal of entry for microbes to both the gastrointestinal and respiratory tracts, as this will allow us to understand what constitutes microbial communities in health and disease [4].

Fungi are ubiquitous organisms that colonize humans, animals, fruits, vegetables, and other plant material. Some fungi inhabit humans without any harmful effects, for example, the symptom-free oral carriage of *Candida* species

that has been recognized for many years [5]. On occasion, yeast can be problematic in immunosuppressed patients [6,7]. The shift from innocuous commensals to harmful pathogens may depend on factors other than the microorganism's attributes. Local or systemic predisposing factors in the host may be of equal or greater importance in the pathogenesis of the disease [5,8].

Our current understanding of oral microbiology is focused on bacteria and *Candida* species. The vast majority of oral microbiology studies use techniques that favor the isolation of bacteria. Consequently, the fungi recovered in these studies are often considered to be contaminants. When studies specifically focus on the isolation of oral yeast and/or moulds, generally one finds a much higher recovery of fungi when compared with surveys that look for bacteria [3,9].

In recent multitag pyrosequencing (MTPS) studies, Ghannoum *et al.* [3] recognized numerous unidentified fungal species as components of the human oral microbiome. In MTPS, multiple samples are bar coded using tagged fusion primers; dozens of samples are then run at the same time. In addition, this technique identifies either viable or nonviable microorganisms. Pyrosequencing allows a global mycobiome characterization independent of culture methods. However, this approach is very expensive and requires very complex data analysis, so it is currently not practiced by most investigators and dentists. Although culture methods can result in incomplete recognition of the entire oral fungi community, they offer relevant information on microbial viability and its association with oral health and disease.

The purpose of this investigation was to characterize the oral fungal microbiota, that is, yeast and moulds, of healthy young participants over time using an improved culture method [10]. Much is known about bacterial microbiome, but fungi, which are probably as important as bacteria in oral colonization, have to date been overlooked. As with typical pathogens such as bacteria and *Candida*, colonization by other fungal species can be the first step to opportunistic infections.

Methods

Study participants

Initially, 40 healthy students in their fifth year of the master's degree program in the Dental Medicine Faculty, University of Porto, Portugal, were invited to voluntarily participate in the investigation. The ethics committee of the same faculty approved the consent form and research protocol. Exclusion criteria included parafunctional habits that could influence the oral microbiota (such as nail biting, finger sucking, and mouth breathing), probing depths >3 mm and/or retraction, and antibiotic or steroid therapy in the last 6 months.

The medical and dental histories of each participant as well as information regarding oral hygiene habits, alcohol habits, and use of contraceptive drugs were obtained by interview. Oral clinical examination included assessment of probing depth, gingival retraction, and bleeding on probing at six sites/teeth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual) for all teeth. The prevalence of caries was assessed by DMFS (decayed, missing, and filled surface) index, and oral hygiene was assessed by plaque control record [11].

Sample collection and cultivation

Oral samples were collected at least 1 h after a meal or tooth brushing, between 11 a.m. and 1 p.m., to avoid variation in salivary flow rates. Participants were asked not to use mouthwash during the week prior to sampling. To assess individual variation of oral fungi colonization over time, a follow-up of 10 participants was carried out 28 weeks and 30 weeks after the first sampling. This allowed for collection of samples within longer (6 months) and shorter (15 days) time periods. The selection criteria for the follow-up participants included continued participant availability and continued residence in the area (Porto, Portugal).

Study participants rinsed their mouth with 15 ml sterile water for 15 s; then 10 ml of the collected samples was diluted in 250 ml of Sabouraud glucose agar supplemented with 50 mg/l of chloramphenicol in a vertical laminar flow hood. The culture media were immediately divided among 10 petri dishes using the pour plate method. Five dishes were incubated for 7 days at 25°C and the remaining five were incubated at 37°C. The cultures were examined every day, with the number of fungal colonies recorded in colony-forming units per milliliter (CFU/ml) and the growing fungi identified at a genus and species level (whenever possible) based on macro- and microscopic morphology [12]. We also used the API system (API/ID32C) for yeast identification, according to the manufacturer's procedures (bioMérieux, Marcy L'Etoile, France). All fungi that were unable to be reliably identified based on traditional features were classified as nonidentified moulds or yeast. The most prevalent nonidentified moulds were subsequently identified using molecular techniques, mainly by sequencing 18S and internal transcribed spacer (ITS) regions of ribosomal RNA genes. The following criteria were established for *Candida* colony counts: patient whose sample values were <50 CFU/ml were considered low yeast carriers; patient whose sample values were between 50 and 400 CFU/ml were considered moderate yeast carriers; and patient whose sample values were >400 CFU/ml were considered high yeast carriers [13].

Table 1. Demographic characterization and oral health evaluation of participants, including the initial sample ($n = 40$) and the follow-up sample ($n = 10$).

Participants characterization	Initial sample	Follow-up sample	*P* value
Age, years	24.0 ± 2.8	23.4 ± 1.6	0.542
Oral hygiene index, %	45.1 ± 12.1	42.3 ± 11.0	0.455
Decayed, missing and filled surface	7.5 ± 7.3	4.1 ± 3.1	0.253
Probing depth, mm	2.1 ± 0.4	1.9 ± 0.3	0.264
Bleeding on probing, %	4.8 ± 4.4	5.7 ± 5.8	0.839

Values are mean ± standard deviation. *P* values were calculated using Mann–Whitney U test to acknowledge differences between values in initial sample and follow-up sample.

DNA extraction, amplification, and sequencing conditions

DNA was extracted from conidia with a sodium hydroxide methodology that is available online (http://www.aspergillus.org.uk/indexhome.htm?secure/laboratory_protocols/index.php~main). DNA (50–250 ng) was suspended in 50 µl of sterile water and stored at −20°C. Genomic regions, 18S and ITS (ITS1 and ITS2), were amplified using a set of the primers described previously [14,15]. Amplification polymerase chain reactions (PCRs) were performed using 1 µl of genomic DNA (1–5 ng/l), 2.5 µl of Qiagen multiplex PCR master mix (Qiagen, Hilden, Germany), and 0.5 µl of each primer (final concentration of each primer was 0.2 µM), in a final volume of 5 µl. After a 15-min preincubation step at 95°C, PCRs were performed for 30 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 90 s, and extension at 72°C for 1 min, with a final extension step of 10 min at 72°C. Next, the PCR-generated fragments were purified on Microspin S-300 HR columns (Amersham Pharmacia Biotech, Quebec, Canada). The sequencing reaction included a 2-min 95°C preincubation step, PCR for 35 cycles with denaturation at 96°C for 15 s, and annealing at 50°C for 9 s, 60°C for 2 min, and 60°C for 10 min. Sequencing reactions were carried out using the ABI Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Division Headquarters, San Francisco, CA, USA). The products were purified using AutoSeqG-50 columns (Amersham Pharmacia Biotech, Quebec, Canada). The DNA fragments were dissolved in 8 µl of HiDi formamide and separated using an ABI PRISM 3100 genetic analyzer 16-capillary electrophoresis system (Applied Biosystems, Division Headquarters, San Francisco, CA, USA). The results were analyzed using Sequencing 5.2 analysis software (Applied Biosystems, Division Headquarters, San Francisco, CA, USA).

Data analysis

The categorical variables were described as relative frequencies (%), and continuous variables were described using mean ± standard deviation. Comparisons between categorical and continuous variables were performed with the χ^2 test and Mann–Whitney U test, respectively. The nonparametric Friedman test was used to analyze changes in quantitative data over time. Correlations between variables were evaluated by the Pearson test using 2-tailed *P*. A *P* value of 0.05 was considered significant. The analyses were performed using the statistical analysis program SPSS version 17.0 (Statistical Package for Social Sciences).

Results

Participant demographics

The initial participants included 26 females (65%) and 14 males (35%), and the follow-up test population was comprised of 9 females (90%) and 1 male (10%). Table 1 shows the demographic characterization and oral health evaluation of the participants. No differences were found between the initial and follow-up sets of individuals regarding demographic characterization and oral health.

Characterization of oral fungi

The recovery rate for oral fungi was higher when the incubation temperature was 25°C compared with 37°C. One hundred percent of samples yielded moulds and 92.5% yielded yeasts when cultures were incubated at 25°C, whereas 42.5% yielded moulds and 45% yielded yeasts when incubated at 37°C.

The most common isolated fungi from samples were *Candida* (67.5%), *Rhodotorula* spp. (75%), *Penicillium* spp. (85%), *Aspergillus* spp. (75%), *Cladosporium* spp. (72.5%), *Trichoderma* spp. (10%), *Scedosporium* spp. (7.5%), *Alternaria* spp. (5%), and *Rhizopus* spp. (2.5%). Twenty percent of the participants were "moderate *Candida* carriers," with samples yielding from 50 to 400 CFU/ml (Fig. S1). The yeasts identified in these participants were *C. albicans, C. parapsilosis*, and *C. tropicalis*. In contrast to that observed with yeast, the level of moulds recovered was similar between the study participants,

Table 2. Overall distribution of the most prevalent fungal genera in the saliva of each of 40 participants

Patient number	Candida	Rhodotorula	Penicillium	Aspergillus	Cladosporium	Alternaria	Trichoderma	Scedosporium	Rhizopus
1	•		•					•	
2	•		•	•					
3	•								
4	•	•	•	•	•				
5			•		•				
6	•	•	•	•	•				
7		•	•	•	•				
8	•		•	•		•			
9			•	•					
10	•	•	•		•		•		
11	•	•	•						
12	•	•	•	•	•				
13	•			•					
14	•			•					
15	•	•		•			•		
16	•	•	•	•			•	•	
17	•	•	•	•	•		•		•
18	•	•	•	•	•				
19		•	•	•	•				
20			•	•	•				
21		•	•	•	•				
22	•	•	•		•				
23	•	•	•	•	•				
24	•	•	•	•	•				
25		•	•	•					
26	•	•	•	•	•				
27	•	•	•	•	•				
28	•	•	•	•	•				
29	•	•	•	•					
30	•				•				
31	•	•	•	•	•				
32		•	•	•	•	•			
33		•	•	•					
34		•	•	•	•		•		
35	•	•	•		•			•	
36		•	•	•	•				
37		•	•	•	•				
38	•	•	•		•				
39		•		•	•				
40	•	•	•	•	•				
Total	27	30	34	31	27	2	5	3	1

• indicates presence of the fungal taxon. Bottom line indicates the number of participants with the fungal genera.

ranging from 1.6 to 8.6 CFU/ml (mean of 4.5 CFU/ml, as shown in supplementary Tables S1 and S2). There was a high prevalence of *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* spp., with values ranging from 0.2 to 5.0 CFU/ml, 0.2 to 5.6 CFU/ml, and 0.4 to 4.6, respectively (Table S1). Regarding *Aspergillus* spp., *A. fumigatus* was found in 40% of all participants, but other species such as *A. flavus* and *A. glaucus* were also sporadically isolated. In addition, molecular identification revealed few atypical colonies of *Penicillium* and *Cladosporium*. The total fungi concentration ranged from 3.4 to 344.2 CFU/ml (mean of 40.4 CFU/ml; Table S1).

The interindividual analysis of oral fungi showed a considerable variability of taxa colonizing the study participants. In Table 2, 23 profiles of the most prevalent fungi among 40 studied individuals are listed. The interindividual variability of the fungal profiles is evidenced by comparing the number of different taxa colonizing each individual with the mean number of taxa colonizing the global study population (χ^2 test, $P = 0.000$). This analysis takes into

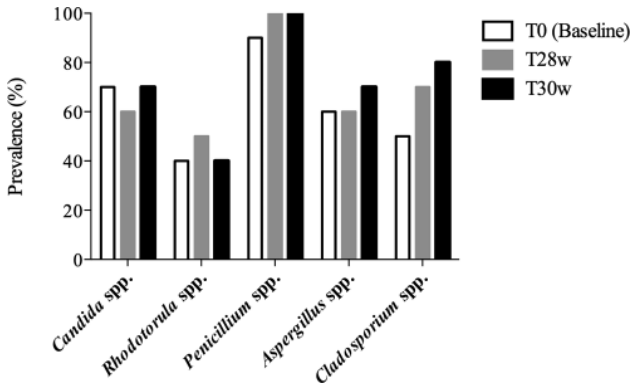

Figure 1. Frequency of the most prevalent fungi at baseline (T0), 28 weeks (T28w), and 30 weeks (T30w) after recovery at 25°C.

account the nine fungal taxa identified. However, this interindividual variability is maintained even when the analysis is performed only with the five most frequent fungal taxa ($P = 0.019$).

On the other hand, an intraindividual stability over time was observed regarding prevalence and quantity of oral fungi. When the follow-up population was viewed collectively, the level of fungi was constant over the 30-week observation period, either after longer (28 weeks after baseline) or shorter (30 weeks after baseline) time intervals, both at 25°C and 37°C (Table S2). In addition, the prevalence of the most frequently recovered fungi was constant throughout the study (Fig. 1). When the study population was viewed individually, it was possible to observe that the participants were systematically colonized by the same fungal taxa at the same concentrations during the three sampling periods. Figure 2 shows fungal quantification over the 30-week observation period; Figure 2A shows results for *Candida* spp. and *Rhodotorula* spp., and Figure 2B shows results for the most prevalent moulds.

Variables such as age, sex, use of oral contraceptive drugs, and alcohol habits did not affect the prevalence and concentration of the fungi observed in the oral cavity ($P < 0.05$). Also, no correlation was found between the oral hygiene or dental caries indexes and the prevalence of *Candida* spp., *Rhodotorula* spp., *A. fumigatus*, or *Trichoderma* spp. ($P < 0.05$).

Discussion

In the present study, we found oral fungal colonization in all participants and, interestingly, a large interindividual variability but a consistent intraindividual stability of fungal taxa diversity and concentrations over time. Cultivable oral fungi included a variety of moulds (mainly *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* spp.) and yeast (especially *Candida* spp. and *Rhodotorula* spp.). Our results were consistent with those obtained recently by Ghannoum *et al.* [3], who analyzed the fungal component of the oral microbiota using MTPS and reported several cultivable fungi, including yeast and moulds. Both studies support the role of a fungal community, more diverse than previously thought, on the equilibrium of oral microbiota.

According to the literature [3,16], *Candida* spp. are the most important and prevalent colonizing fungi within the oral mucosa, as they are associated with oral diseases, especially in immunocompromised hosts [17]. The overall frequency of yeast carriage determined in the present study was 92.5%, which was much higher than that previously reported, that is, carriage ranging from 30% to 75% [3,16]. Of the individuals in this study, 20% showed fewer than 5 yeast/ml of saliva. It should be noted that lower recovery levels could not be detected using standard microbiological culture techniques. Moderate yeast carriage was observed in 20% of the participants; patients with clinical signs of oral candidosis usually had more than 400 CFU/ml [13]. None of the participants presented with more than 400 CFU/ml or signs of candidosis.

The filamentous fungi identified in oral samples were mostly ubiquitous moulds. The most prevalent were *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* spp., which are among the most frequent airborne moulds found indoors and outdoors [18,19]. The overall frequency of mould carriage was very high (reaching 100%). These values can be attributed to the sampling and culture methodologies used in this study for fungi recovery. We used a pour plate method, which is more sensitive for detection and quantification of yeast and moulds than the commonly used direct spread plate technique. In addition, two incubation temperatures were used (25°C and 37°C), with a higher fungal recovery rate observed at 25°C. Despite this, incubation at 37°C, the most common temperature used in oral microbiology studies, was better for recovery of pathogenic yeast and moulds. However, at 37°C, some of the fungal biodiversity was lost. The optimum growth temperature for most fungi is below 30°C, even for some pathogenic fungi such as the dermatophytes [12,18].

The observation of moulds in cultures from oral samples is frequently interpreted as contamination due to airborne spores. In the current study, all samples were managed in a vertical laminar flow hood and environmental contamination was carefully avoided. The stability of fungal concentrations and taxa observed over time among the participants supports the assumption that contamination was rare in our sample management. Hence, it can be assured that all the isolated fungi were colonizers of the oral cavity of tested individuals. Their presence in the oral mucosa is not unusual, as they have been frequently identified in the nasal mucosa [20,21]. Our results are consistent

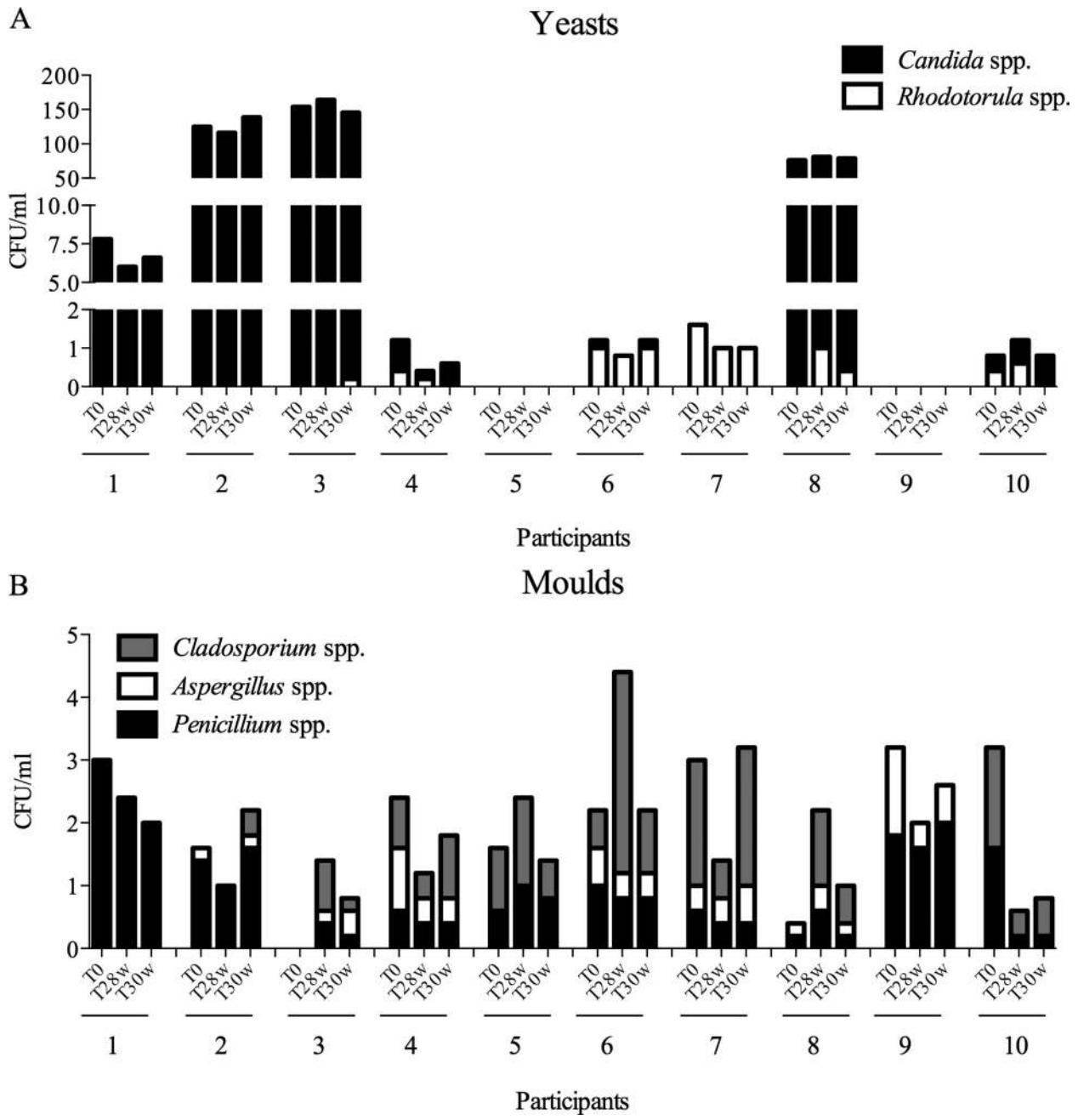

Figure 2. Individual colonization of yeasts (A) and most prevalent moulds (B) at baseline (T0), 28 weeks (T28w), and 30 weeks (T30w) after recovery at 25°C.

with those published by Buzina *et al.* [20], in which the biodiversity of fungi isolated from the patients' nasal mucus was characterized over 28 months, and it was found that the most prevalent isolates belonged to the genera *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, and *Aureobasidium*. In a second study performed by Ponikau *et al.* [21], the most prevalent genera were *Cladosporium*, *Alternaria*, *Aspergillus*, and *Penicillium*.

The clinical relevance of this newly identified oral mycobiome is still unknown for either healthy participants or immunocompromised hosts. Similar to what typically happens with bacteria and *Candida*, colonization with a given fungal species could be the first step toward an opportunistic infection. In the last two decades, fungi have emerged as a major cause of human disease, especially in immunocompromised or critically hill hosts [22]. Most invasive fungal infections are caused by *Candida* spp., which continue to be the fourth most common microorganism recovered in bloodstream infections in the United States [23], while *Aspergillus* spp. are the second most common cause of

invasive mycosis [24]. In the present study, we found a high prevalence of oral *Candida* spp. (92.5%) and *Aspergillus* spp. (75%). We also found that *A. fumigatus*, the most common cause of invasive aspergillosis [22,25], was the most frequent *Aspergillus* species, found in 40% of all participants. In the study by Perfect *et al.* [26], a single culture positive for *Aspergillus spp*. was considered predictive for invasive disease in high-risk patients. Given the high prevalence of oral fungi colonization found in the present study, as well as the persistence of these microorganisms over the 30-week time frame, it would also be pertinent to follow this population throughout life in order to understand their risk for invasive mycosis.

The Human Microbiome Project aims to characterize the human microbiome and its role in health and disease [27]. The present study increases our knowledge regarding the oral fungal microbiota, which may have implications for the future of dental practice, particularly with regard to antimicrobial therapy. As reported for oral bacteria [1,28,29], we demonstrated that oral fungi present a great interindividual variability but a consistent intraindividual stability over time, suggesting a common and a variable group of fungi in the oral cavity. Of particular interest was the intraindividual stability observed over a period of 30 weeks that included a seasonal climate change. Sampling was performed at long and short time gaps of 28 weeks and 2 weeks in order to evaluate the stable or transient character of oral mycobiome.

In conclusion, we found that all individuals included in this study showed evidence of oral fungal growth, with a prevalence of 100% for moulds and 92.5% for yeast, and that the oral fungal community presented high interindividual variability but consistent intraindividual stability over time.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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