Yeast diversity in the oral microbiota of subjects with periodontitis: *Candida albicans* and *Candida dubliniensis* colonize the periodontal pockets

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The term periodontitis encompasses several polymicrobial infectious diseases, of multifactorial etiology, with chronic and aggressive forms. In spite of the etiopathogenic differences between these two forms of the disease, few studies have analyzed the subgingival colonization by yeast. The objective of this investigation was to analyze the composition of the yeast microbiota present in the mucosa and subgingival sites of healthy individuals and patients with aggressive and chronic periodontitis. For this, samples were recovered from these two locations and the yeast recovered identified by phenotypic and genotypic methods. Patients with chronic periodontitis showed significant differences in relation to the other groups with respect to carrier status (69.2% versus 35.7% of healthy individuals; [χ_i^2 test; $p = 0.014$]), the total number of isolated colony forming units or CFU (mean and ranges 281.6 (0–6048) [K-W$_2 = 6.998$; $p = 0.03$]), the Simpson diversity index (*I*) in site *b* ($I_b = 0.344$ versus healthy subjet and aggresive periodontitis where $I = 0$ [multiple *t*-test comparisons with the Bonferronni correction, $p < 0.05$]), and the species profile. Interestingly, in spite of the varied profiles of the species present in the mucosa of the three groups analyzed we noted that only *C. albicans* and *C. dubliniensis* were capable of colonizing the periodontal pockets in patients with chronic periodontitis, while only *C. albicans* was identified in the subgingiva of healthy individuals and patients with aggressive periodontitis.

Keywords   Candida, yeast diversity, chronic periodontitis

Introduction

Periodontal disease is a chronic inflammation characterized by the destruction of support connective tissue and alveolar bone loss with the formation of a periodontal pocket [1,2].

Chronic periodontitis (CP) has both localized and generalized forms. It is initiated and sustained by a polybacterial infection caused by *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* among other species. In addition, host defense mechanisms are also important in the pathogenesis and tissue destruction [3–5]. CP, the most frequent form of periodontitis, begins at any age although it is more common in adults, and is characterized by its extension (number of sites involved) and severity (amount of clinical insertion loss) [1,6]. Aggressive periodontitis (AP) also presents localized and generalized forms which are characterized by the rapid loss of bone and insertion and the presence of

familial aggregation. Localized AP which begins close to puberty, is characterized by the presence of abundant seric antibodies against infectious agents (*A. actinomycetemcomitans*), and has a specific distribution in teeth of the dental arches. The generalized form affects individuals over 30 years of age with a low antibody response and a sporadic and pronounced loss of bone and insertion in at least three permanent teeth [1,7]. Furthermore, the frequency of cases related to *A. actinomycetemcomitans* decreases and the number associated to *P. gingivalis*, *T. forsythia*, *Prevotella intermedia*, *Dialister pneumosintes*, *Campylobacter rectus*, species of *Fusobacterium*, *Selenomonas sputigena* and spirochaetes increases [8–10].

Results of epidemiological studies have shown that periodontal disease has both a high prevalence and severity in the world [11–15]. In the Chilean population, 100% of subjects between the ages of 65–74 are affected, while 10% of adolescents between the ages of 15–19 suffer the disease [16]. In spite of the polymicrobial character of periodontal disease, the role of yeasts in this illness has received little or no attention [2,17–23].

The presence of yeasts colonizing different regions of the human body, including the oral cavity has been associated with both healthy and ill individuals [24–26]. It is estimated that around 40% of healthy people care members of the genus *Candida* in saliva or oral mucosa [27–29]. In the healthy carrier, various local and/or general predisposing factors confer *Candida* the capacity to invade different mucosal tissues, making it an opportunistic pathogen [24–26,30]. *Candida albicans* is the main species responsible for the majority of mycotic infections in the oral cavity, although other members of the genus may be involved [24,30].

In conditions of oral health, yeast of the genus *Candida* may be present on the palatine and vestibular buccal mucosa, on the mouth floor, tongue and saliva, but rarely in the subgingiva [28,31]. However, they may be isolated from the subgingival microbiota in about 17% of patients with periodontitis [2,17,20,22,32]. Furthermore, it has been reported that the proportion of yeasts in the periodontal pockets is similar to some bacterial periodontopathogens, suggesting a possible role for *Candida* spp. in the pathogenesis of the disease [10,17,33].

In spite of the involvement of different etiological agents in chronic and aggressive periodontal disease, few studies analyze the possible role of yeasts in any of these forms of periodontitis [18,20,22,34]. Therefore, the majority of studies that report the presence of yeasts in periodontal pockets do not specify if patients suffer the chronic or aggressive form of the disease [2,17,21–23,31,32,34–36]. One of the few works that specified the subtype of periodontitis did not provide information on non-carriers in a study of 25 patients with CP and 1 with AP [19]. Another recent investigation reported a prevalence of 16% of the yeast *Candida albicans* in 25 patients with CP but did not include subjects with AP [20]. Furthermore, a study that analyzed biopsies obtained from 12 patients with juvenile periodontitis did not include patients with CP [18].

In spite of the role suggested for *Candida* spp. in periodontitis, it is currently unclear if yeasts participate in the etiology of this disease and if they show specificity for either the chronic or aggressive forms [2,18,20].

In the present study, the composition of the yeast microbiota present in mucosa, sulcus and subgingival sites of healthy subjects and patients with AP and CP was analyzed, with the purpose of describing possible differences between these groups of individuals with respect to their periodontal condition.

Materials and methods

Individuals

Periodontally healthy (PH) subjects. Twenty-eight volunteers without periodontal disease or other systemic complications participated in the investigation. The state of periodontal health was determined by the following clinical characteristics of the gum; pale pink coloration, firm tissue consistency, festooned contour of the gingival margin, firm dental papillae that fill the space under the contact areas, absence of inflammation and/or bleeding to soft probing. In addition, we confirmed the absence of periodontal pockets and loss of clinical insertion of tissue associated with infection and/or sites with probing depth of greater than 3 mm.

Patients with aggressive periodontitis (AP). The 20 patients within this group were selected according to the following criteria; estimation of the age of onset by medical examination and interview of <30 years old, ≥ 4 mm loss of attachment on more than two first molars and/or incisors, and three or more affected cuspids, premolars, or second molars [37].

Patients with chronic periodontitis (CP). This group was composed of 26 individuals selected according to the following criteria; ≥ 4 mm loss of attachment observed in at least 30% of residual teeth [37].

The individuals were duly notified of the nature of the investigation, which was approved by the ethics committee of the Faculty of Dentistry (University of Chile, Santiago, Chile), and all signed an informed consent forms. All the subjects fulfilled the following general criteria; no history of systemic diseases, pregnancy, breastfeeding, immunodepression, antibiotic treatment, antimycotic and anti-inflammatory drugs in the 6 months prior to the study, previous periodontal treatment, use of orthodontic apparatus, use of partial and/or total prosthesis and presence of local and/or systemic factors that predispose candidiasis. The incidence of smoking was also considered. At the beginning of the study one of the investigators (GJ) determined the plaque index, bleeding percentage and subsequently carried out supragingival prophylaxis to remove the tartar and facilitate the clinical exam of the periodontal tissues.

Yeast sample

In the PH subjects, samples from the oral mucosa and subgingiva of two mesiobuccal sulcus (pieces 16 and 26) were taken. The samples of oral mucosa were collected using small sterile cotton rolls and pooled from the internal cheek face and from the third half of the dorsal side of the tongue. The samples from the subgingiva were obtained by introducing a paper cone in the sulcus of both pieces for 10 seconds, pooled and deposited in tubes with 2 ml PBS buffer pH 7.4 (137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4) and transported to the laboratory at 4°C for analysis.

In patients with periodontitis, the samples were taken from oral mucosa following the procedure described previously. Material from the subgingiva was obtained from three periodontal sites, i.e., less than 3 mm (site *a*), 4–6 mm (site *b*) and more than 6 mm depth (site *c*). In taking samples, teeth were isolated using cotton rolls, the supragingival bacterial plaque was removed and the gingival site was carefully dried with a syringe prior to the insertion of a paper cone for 10 sec. The samples of each site were placed in separate tubes containing PBS buffer.

Processing of samples

The samples were concentrated by centrifugation at 17,320 *g* for 10 min and resuspended in 300 μl of PBS buffer. The total volume was inoculated onto one Sabouraud dextrose agar plate with tetracycline (50 μg/ml) and incubated at 37°C for 48 h. The colonies were recovered, counted and stored on independent plates for their identification.

Yeast identification

Identification of the isolates was accomplished through conventional tests such as formation of germination tub and microculture [38], as well as the use of Chromoagar *Candida* [39]. In order to differentiate *C. albicans* and *C. dubliniensis* strains, the yeasts were grown on Sabouraud dextrose agar at 42°C and in media containing xylose [39,40]. When needed, Fungichrom and API ID 32C were used according to the manufacturer's instructions [41,42]. When conventional identification test results were ambiguous, the information was supplemented by sequencing the ITS1-5.8S rDNA-ITS2 region using the primers ITS1 and ITS4 [43]. The BigDye kit (Applied Biosystems) and an automated DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems) were used for sequencing. Sequences were compared against a public DNA sequence database using BLAST algorithms [43].

Statistical analysis

The differences in the group averages for the age variable were determined using the ANOVA and Tukey tests. The group differences relative to gender and smoking variables were compared using the Chi-square test. The differences in the group means for the clinical variable pocket depths, insertion level, plaque index and bleeding percentage were compared using the Student *t*-test. The differences in the yeast carrier status between the sites and between groups of individuals were evaluated using the Chi-square test. The differences in number of the colony forming units (CFU) present in mucosa and subgingiva in the three groups were evaluated using the Kruskall-Wallis test. The association between the periodontal condition and the colonizing species was determined by a correspondence analysis, using the number of CFU obtained for each species in the three groups of subjects as a variable. This analysis establishes the force of the association between yeast species and the periodontal status using the value of a coefficient named inertia, which is associated to the Chi-square distribution. The computer programs used were STATISTICA® Version 6.0 and STATA® version 8.0.

Results

Clinical characteristics of the patients studied

The group of PH subjects composed of 18 women and 10 men had an average age of 27.9 ± 6.7 years. In contrast, the group of patients with AP had 13 women and seven men with an average age of 28.7 ± 6.9 years

and the group of patients with CP had 18 women and eight men whose average age was 40.8 ± 10 years.

From a clinical periodontal point of view, patients with AP had an average plaque index of 50.9% and patients with CP had an index of 61.5%. In these groups of patients, the average bleeding percentage was 40.5 and 55.4%, respectively. The pocket depth was 3.5 mm and 3.3 mm, while the average level of clinical insertion was 3.5 mm and 3.4 mm in patients with AP and CP, respectively. There were no significant differences in the percentage of individuals who smoked among the three groups studied. As a result of the age distribution of the illnesses, the statistical analysis revealed that there were differences in the average age among the groups analyzed ($F_{2,71} = 21.136$; $p = 0.001$). These differences were found between the PH subjects and patients with CP (Tukey Test; $p < 0.001$) and patients with AP and CP (Tukey Test; $p < 0.001$). Furthermore, differences in the plaque index ($t_{44} = 2.51$; $p = 0.0159$) and bleeding percentage ($t_{44} = 3.02$; $p = 0.0041$) were found between AP and CP patients.

Yeast carrier status

Considering all the individuals in each group, it was possible to observe that the most frequently colonized anatomic site was the mucosa (Table 1). Hence, of a total of 10 carriers in the PH group, 100% showed yeast colonization at this site but only one of these subjects (10%) was colonized in the subgingiva. In the nine patients with AP, 100% had yeasts in the mucosa, while only 4 (44%) had yeast in the subgingiva. Finally, of the 18 patients with CP, 17 (94.4%) had yeast in the mucosa and eight (44.4%) were colonized in the subgingiva. Furthermore, a significant difference was observed in the number of total carriers in the CP group (69.2%) with respect to the PH subjects (35.7%) [χ_i^2 test; $p = 0.014$].

Number of yeast isolated per group

To analyze if the degree of yeast colonization present in the various sites sampled showed differences among the three groups, the CFUs (colony forming units) per subject of yeasts recovered from mucosa and subgingiva samples were determined (Table 2). In general terms, the results were variable and showed a heterogeneous distribution with a broad range. Furthermore, in mucosa as well as subgingival sites, the PH subject group was found to have the lowest CFU ranges (0–62 and 0–5, respectively). Of the affected individuals, the group of patients with AP showed intermediate ranges (0–194 and 0–100), while the group of patients with CP showed the highest (0–2140 and 0–6048). It is important to highlight that, although it was the same subject with CP who showed the highest CFU values in both sites, the other individuals from this group also showed consistently higher colonization values than PH subjects and those with AP (data not shown). Moreover, when the CFUs obtained from mucosa and subgingiva sites in the three groups was analyzed separately using the Kruskall-Wallis test, significant differences were found among the three groups relative to the subgingival sites but not in the mucosa (Subgingiva: Kruskal-Wallis$_2$ = 6.998; $p = 0.03$ and Mucosa: Kruskal-Wallis$_2$ = 4.9; $p = 0.086$).

Distribution of yeast species per individual and species association

Table 3 shows the prevalence, yeast species profile and species diversity index (I), in mucous and subgingival sites of each of the PH subjects and patients with AP and CP. In both anatomical sites, most of the individuals showed only one yeast species. Two yeast species were recovered from the mucosa of two PH individuals, three subjects with AP and three with CP and one individual with CP had three species. At the periodontal level, only one PH subject and all with AP

Table 1 Number of yeast carriers in mucosa and subgingival sites in periodontally healthy (PH) subjects, patients with aggressive periodontitis (AP) and chronic periodontitis (CP).

Group	Frequency and (%) of carriers in:			
	Only mucosa	Only subgingival sites	Mucosa and subgingival sites	Total carrier frequency and (%)
PH subjects ($n = 28$)	9 (32.1)	0 (0)	1 (3.6)	10 (35.7)
AP patients ($n = 20$)	5 (25.0)	0 (0)	4 (20.0)	9 (45.0)
CP patients ($n = 26$)	10 (38.5)	1 (3.9)	7 (26.9)	18 (69.2) †

†Statistical difference between the total percentage of yeast carriers in patients with CP and PH subjects.

Table 2 Means and ranges of the number of CFU (colony forming-unit) of yeast isolated from mucosa and subgingival sites in periodontally healthy (PH) subjects and patients with aggressive periodontitis (AP) and chronic periodontitis (CP).

Group	Mucosa Mean (range)	Subgingival* sites Mean (range)
Control ($n=10$)	4.7 (0–62)	0.18 (0–5)
Aggressive P. ($n=9$)	17.5 (0–194)	12.2 (0–100)
Chronic P. ($n=18$)	99.6 (0–2140)	281.6 (0–6048)

*Statistical difference in the number of CFU present in subgingival sites in the three groups analyzed by the Kruskal-Wallis Test ($K\text{-}W_2 = 6.998$; $p = 0.03$).

showed one yeast species. In the cases of patients with CP, one individual had two yeast species in site *a* and two subjects in site *c* (Table 3). The yeast species found in mucosa of PH subjects were *Candida albicans/Saccharomyces cerevisiae* and *Candida dubliniensis/Candida guillermondii*. In the case of the AP patients, the yeasts in the mucosa were *Candida albicans/Candida guilliermondii* and *Candida albicans/Candida glabrata,* while with CP patients *Candida glabrata/Candida albicans, Candida dubliniensis/Saccharomyces cerevisiae, Candida albicans/Candida zeylanoides,* and *Candida dubliniensis/Candida guilliermondii/Candida albicans* were found. In subgingival sites of patients with CP the following two yeasts were noted, *Candida albicans/Candida dubliniensis* and *Candida albicans/Candida glabrata*. Table 3 also shows that in the three pocket depths analyzed, the number of subjects colonized was similar in patients with AP (site $a=2$, site $b=4$ and site $c=3$), while in patients with CP, sites *a* and *c* were slightly higher (site $a=7$, site $b=4$ and site $c=6$).

With respect to the species profile observed in each group, six different species were identified in the mucosa of the PH subjects, i.e., *C. albicans, C. dubliniensis, C. guillermondii, Candida parapsilopsis, Kluyveromyces lactis* and *Saccharomyces cerevisiae*. In the mucosa of patients with AP, we found *C. parapsilopsis, C. albicans, C. glabrata* and *C. guillermondii* and 10 species were identified in individuals affected with CP, i.e., *C. albicans, C. dubliniensis, C. glabrata, C. guillermondii, Candida lusitaniae, C. parapsilopsis, Candida sake, C. zeylanoides, Rhodotorula mucilaginosa* and *S. cerevisiae*, where the latter is the most diverse species profile (Table 3). On the other hand, the species profiles in the subgingival sites were much less varied. Hence, only *C. albicans* was found in the only PH carrier subject and in patients with AP and three species were noted in patients with CP, i.e., *C. albicans, C. dubliniensis* and *C. glabrata* (Table 3). In all the individuals, the species found in the subgingival sites were also

present in the mucosa. Surprisingly, in spite of the varied species profiles found in mucosa in PH subjects as well as in affected individuals, only *C. albicans, C. dubliniensis* and occasionally *C. glabrata* species, were recovered from the periodontal pockets. Interestingly, in the mucosa as well as the subgingival sites of patients with AP, *C. dubliniensis* was not identified (Table 3).

Species diversity per group

Considering the low prevalence of each species found in all the groups, with the exception of *C. albicans*, their diversity in each anatomical site sampled was compared using the Simpson species diversity index [44] (Table 3). In general, the mucosa showed the highest diversity indices. In this site, significant differences were observed among the three groups studied [Multiple *t*-test comparisons with the Bonferroni correction, $p < 0.05$]. The mucosa of patients with AP had the highest diversity index ($I = 0.550$), followed by the mucosa in PH subjects ($I = 0.503$) and patients with CP ($I = 0.339$). The subgingival sites of the PH subjects and patients with AP showed a diversity $I = 0$, as only isolates of *C. albicans* were found. On the other hand, the species diversity in the subgingival sites of patients with CP was significantly different at the different depths of the periodontal pockets [Multiple *t*-test comparisons with the Bonferroni correction, $p < 0.05$] (Table 3). Site *b* showed the highest diversity index ($I = 0.344$), followed by site *c* ($I = 0.197$) and site *a* ($I = 0.004$). Within this same group, when comparing the species diversity index between mucosa and periodontal sites, sites *a* and *c* showed a lower diversity [Multiple *t*-test comparisons with the Bonferroni correction, $p < 0.05$] (Table 3).

Total number of species identified per group

Table 4 shows the total number of CFUs and the number of CFUs identified of each yeast species isolated from mucosa and subgingival sites in each group. In the total number of subjects analyzed ($n = 74$), 11 different yeast species were identified, eight of which belong to the genus *Candida* and three belong to other genera (*Kluyveromyces, Saccharomyces* and *Rhodotorula*).

From the total number of yeast isolates identified (3396) from the three groups, the most prevalent species were *C. albicans, C. dubliniensis* and *C. glabrata*, represented in 87.5%, 8.4%, and 2.6%, respectively, while the rest of the species were found in percentages below 0.5% (Table 4). In the group of PH subjects, 100% of the 136 isolates were identified, and *C. albicans, C. dubliniensis* and *K. lactis* were found to be

Table 3 Prevalence, profile and species diversity (I)*[&] of yeast in the mucosa, sulcus and subgingival sites of different depths in periodontally healthy (PH) subjects, patients with aggressive periodontitis (AP) and chronic periodontitis (CP), respectively.

Mucosa			Subgingival sites						
Periodontally healthy	Aggressive P.	Chronic P.*	Periodontally healthy	Aggressive Peridontitis			Chronic Peridontitis		
N°. of indiv. (Yeast sp.)	N°. of indiv. (Yeast sp.)	N°. of indiv. (Yeast sp.)	Sulcus gingival No. of indiv. (Yeast sp.)	Site a N°. of indiv. (Yeast sp.)	Site b N°. of indiv. (Yeast sp.)	Site c N°. of indiv. (Yeast sp.)	Site a N°. of indiv. (Yeast sp.)	Site b N°. of indiv. (Yeast sp.)	Site c N°. of indiv. (Yeast sp.)
5 C. albicans	3 C. parapsilopsis	5 C. albicans	1 C. albicans	2 C. albicans	4 C. albicans	3 C. albicans	6 C. albicans	2 C. albicans	3 C. albicans
1 C. parapsilopsis	2 C. albicans	2 C. dubliniensis					1 C. albicans/ C. dubliniensis	2 C. dubliniensis	1 C. dubliniensis
1 C. guilliermondii	1 C. guilliermondii	1 C. glabrata							1 C. dubliniensis/ C. albicans
1 K. lactis	2 C. albicans/ C. guilliermondii	1 C. parapsilopsis							1 C. albicans/ C. glabrata
1 C. albicans/ S. cerevisiae	1 C. albicans/ C. glabrata	1 C. sake							
1 C. dubliniensis/ C. guilliermondii		C. lusitaniae							
		z. cerevisiae 1 R. mucilaginosa 1 C. glabrata/ C. albicans 1 C. dubliniensis/ S. cerevisiae 1 C. albicans/ C. zeylanoides 1 C. dubliniensis/ C. guilliermondii C. albicans							
$I = 0.503$	$I = 0.550$	$I = 0.339$	$I = 0.0$	$I = 0.0$	$I = 0.0$	$I = 0.0$	$I = 0.004$	$I = 0.344$	$I = 0.197$

*The species diversity was calculated using the Simpson diversity index, the formula of which (I) and standard error (SE) are shown below, where **s** corresponds to species; **ni** to the i species frequency; **N** is the total number of isolates and **p_i** is the proportion of the i species. The species diversity varies from 0, when all the isolates are of the same species, to a maximum value of 1 when each isolate is of a different species.

[&]Statistical difference in the species diversity indices compared berween and within groups by the *t*-test with the Bonferroni correction, $p = <0.005$.

$$I = 1 - \sum_1^s \frac{n_i(n_i - 1)}{N(N - 1)} \qquad SE = \sqrt{V} = \sqrt{\frac{\sum_1^s p_1^3 - \left(\sum_1^s p_i^2\right)^2}{0.25 \cdot N}}$$

Table 4 Number of yeast identified by species and total number of yeast isolated (CFU) from the mucosa and subgingival sites in periodontally healthy (PH) subjects and patients with aggressive periodontitis (AP) and chronic periodontitis (CP).

Species/group	Periodontally healthy	Aggressive periodontitis	Chronic periodontitis	Total	% of total isolates identified by species
C. albicans	95	552	2324	2971	87.5
C. dubliniensis	17	–	268	285	8.4
C. glabrata	–	12	77	89	2.6
C. parapsilopsis	4	10	2	16	0.5
K. lactis	13	–	–	13	0.4
C. guillermondii	5	4	2	11	0.3
S. cerevisiae	2	–	3	5	0.1
C. sake	–	–	2	2	0.06
R. mucilaginosa	–	–	2	2	0.06
C. zeylanoides	–	–	1	1	0.03
C. lusitaniae	–	–	1	1	0.03
Total number identified	136	578	2682	**3396**	100.0
Total number isolated	136	592	9910	10638	–
% of yeast identified from the total isolated per group	100.0	97.6	27.1	31.9	–

the most prevalent. In patients with AP, 97.6% of the isolates were identified and the majority was *C. albicans*, *C. glabrata* and *C. parapsilopsis*. From the 27.1% of the isolates identified from the group of patients with CP, the most common were *C. albicans*, *C. dubliniensis* and *C. glabrata* (Table 4). On the other
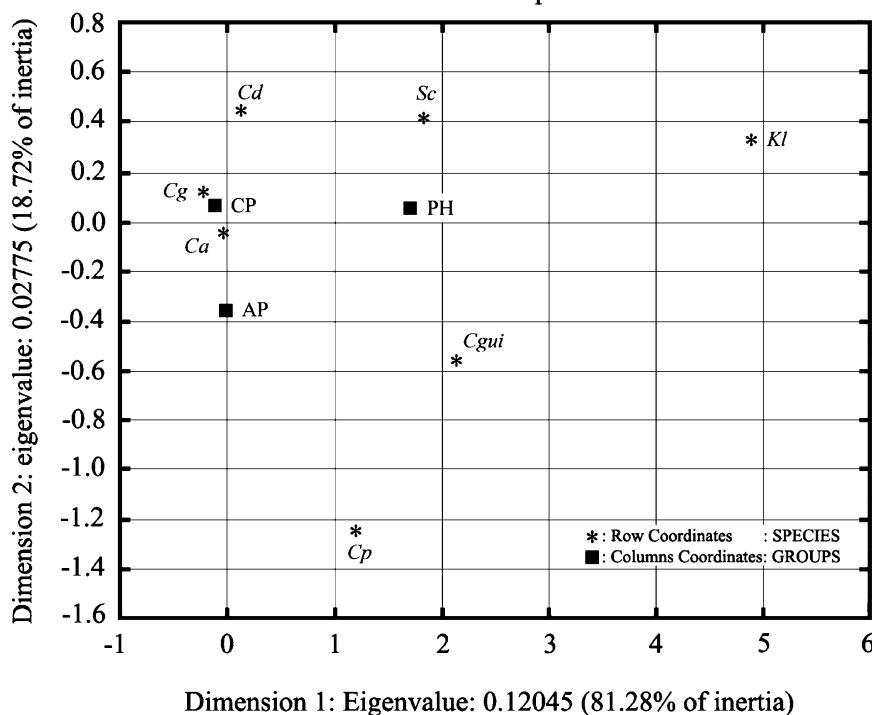

Fig. 1 Compound graph that shows the correspondence between the group of subjects studied and species of colonizing yeast. * =Species, ■ =Groups. PH =periodontally healthy individuals, AP =patients with aggressive periodontitis, CP =patients with chronic periodontitis. *Ca =C. albicans, Cd = C. dubliniensis, Cp =C. parapsilosis, Cgui =C. guilliermondii, Cg =C. glabrata, Sc =S. cerevisiae, Kl =K. lactis.*

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hand, considering the total number of CFUs, patients with CP show a greater degree of colonization than patients with AP and PH subjects.

A corresponding analysis established that there is a strong association between the chronic periodontal condition and some of the colonizing species (Total inertia $=0.148$; $X_{12}^2 = 502.4$; $p \ll 0.001$) (Fig. 1). The PH subjects do not show a close relationship with any species in particular. Patients with AP showed only a slight association with *C. albicans*, but patients with CP were associated with *C. albicans*, *C. glabrata* and *C. dubliniensis* (Fig. 1). Using this same analysis, it can be inferred that *K. lactis*, *C. guillermondii* and *C. parapsilopsis* formed a separate group where none had a preference for a specific periodontal condition (Fig. 1).

Discussion

In this study, the composition of the yeast microbiota recovered from the mucosa and subgingival sites of PH subjects and patients with AP and CP was analyzed. The 32.1% of PH subjects colonized in the mucosa (Table 1) was slightly lower than the 50% reported by Kleinegger [45]. With respect to the subgingival sites of the PH group, only one individual was colonized (Table 1) suggesting that under normal conditions, yeast do not develop easily in the subgingiva.

The majority of published studies on the association of yeasts with periodontitis do not provide information relative to the subtypes of the disease. Several have indicated a 17% prevalence of yeasts in subgingival sites, which is somewhat lower than the 26% found in the total sample of patients in this study ($n = 46$) [2,17,21,22,32]. Of our patients, 32.6% were mucosal carriers, which was similar to that observed in the PH subjects (Table 1). Not all the mucosa carrier patients were also carriers in the subgingival sites, suggesting that the entrance of the yeasts to the sick subgingiva is restricted.

Given the differences in the etiopathogeny of the different subtypes of periodontal disease, and to the bacterial specificity of AP, it appeared necessary to study the yeast microbiota in aggressive and chronic periodontitis separately and contrast the results to what was observed in PH individuals. The percentage of yeast carriers in mucosa in the three groups was similar. On the other hand, although no significant differences were found in the subgingival colonization among the three groups, patients with CP had a greater percentage of colonization than PH subjects. This suggests that subgingival colonization by yeasts could be favored in the chronic periodontal disease (Table 1).

Given the age distribution of patients with both diseases in this investigation, the ages of those with AP and CP were different. Since some reports indicated that the yeast carrier status varied according to age [45], we conducted a logistic regression analysis which showed that this variable was not associated with the difference in the periodontal carrier status. Similarly, it was possible to confirm that smoking was not associated with a major percentage of carriers in subgingival sites in the group of patients with CP.

In the three groups of subjects studied, the distribution of yeast CFUs was very heterogeneous (Table 2), indicating that some subjects were more intensely colonized than others. This agrees with the study by Kleinegger who reported that only 20% of the healthy carriers between the ages of 15 and 60 were intensely colonized [45]. In the present investigation, this characteristic was more common in the group of individuals with CP (Table 2). Additionally, this group of patients had the greatest species diversity index and a greater number of total isolates per species (Tables 3 and 4). These differences could be related to the immunological state of the patients.

Differences were found in the profiles of species that colonized the subgingival sites in both forms of periodontal disease. Hence, the only species identified in patients with AP was *C. albicans*, while *C. albicans*, *C. dubliniensis* and *C. glabrata* were found in patients with CP (Table 3, Fig. 1). Perhaps the conditions of the subgingival sites of the patients with CP are less aggressive for yeast development than the subgingival conditions in patients with AP. However, we cannot discard the role of the immune system in the differences observed between both types of illnesses.

To determine if the depth of the periodontal pockets favored the development of yeast in some of the diseases, isolates were recovered at three different depths of pockets. This is the first report that provides information relative to this variable. The analysis showed that in the three pocket depths studied, the number of AP and CP subjects colonized was very similar. This could suggest that the degree of colonization is not related to the depth of the periodontal pocket in both groups of patients. However, the results indicate that patients with CP showed differences in the profile and in the diversity of species in the periodontal pockets at different depths. The subjects with AP had *C. albicans* at all three depths, while *C. dubliniensis*, *C. glabrata* and *C. albicans* were noted in the CP patient population. Within this same group, the highest diversity index was observed at intermediate depth (Table 3), which could be related to the micro-environmental conditions in this site.

In spite of the varied species profile found in the mucosa of the three groups of subjects analyzed, only *C. albicans*, *C. dubliniensis* and *C. glabrata* were recovered from the periodontal sites. However, the presence of *C. glabrata* in the periodontal pockets could be an exception, since only one of the 18 patients was a carrier of this species in association with *C. albicans* (Table 3). If we consider that *C. albicans* and *C. dubliniensis* are species that share biological and genetic characteristics and methods to differentiate them have only recently been developed, its presence in periodontal pockets could be a relevant finding [46,47].

The mucosal colonization by *C. albicans* and *C. dubliniensis* did not always ensure its presence in the periodontal pockets (data not shown). Although in three subjects with CP where the presence of *C. albicans* was confirmed in the periodontal pockets, the yeast could not be isolated from the mucosa (data not shown). This indicates that to have the global picture of the yeast microbiota in the oral cavity it is necessary to sample both the mucosa and the periodontal pockets. This would also allow the confirmation of our findings of restricted subgingival colonization of the periodontal pockets by *C. albicans* and *C. dubliniensis*.

Interestingly, species associations were found in the mucosa of the three groups studied and in the subgingival sites of patients with CP. However, only *C. albicans* was noted in the subgingival sites of AP patients (Table 3). A possible explanation could be that the periodontopathogens causing AP develop a micro-environment that hinders the co-existence of *C. albicans* with other yeast species, or where only *C. albicans* is capable of surviving given its wide range of virulence factors [22,25,30,48].

A total of 11 different species were recovered from the mucosa, with *C. albicans*, *C. dubliniensis* and *C. glabrata* the most prevalent (Tables 3 and 4). Interestingly, these three species were also found in the subgingival sites of patients with CP. A similar species profile to that found in this work has been reported as being more prevalent in subgingival lesions of HIV carriers [49].

Although not all the yeast recovered were identified, it is important to highlight that 14 isolates (2.4%) obtained from an AP patient could not be identified because they did not remain viable during our identification procedure (data not shown). In this individual, 94.7% of the yeasts identified were *C. albicans*. In patients with CP, 7,228 isolates were unidentified, of which 6,812 colonies (92.4%) were isolated from a single patient. In all the sites analyzed in this indivi-

dual, 1,376 isolates were identified as *C. albicans* and no other yeasts were found (data not shown). Of the remaining unidentified colonies in the CP group (416 colonies), 93 *C. albicans* isolates were recovered from two patients, while 323 unidentified isolates came from only one patient who presented *C. dubliniensis* (97.0%), *S. cerevisisae* (1.5%) and *C. albicans* (1.5%). According to these data, the number of unidentified yeast in Table 4 does not significantly alter the distribution and proportion of each species in the three groups analyzed.

We also report the finding of *C. dubliniensis* in mucosa of PH subjects and in mucosa and subgingival sites of various depths in patients with CP. Although this species has been previously reported in cases of candidemia in Chilean patients [40], this is the first work in our country that describes its identification in periodontally healthy subjects and patients with chronic periodontitis.

The results reported in this work, indicate that there are differences in the characteristics of the yeast microbiota recovered from subgingival samples in patients with CP, when compared to patients with AP and PH subjects. Furthermore, *C. albicans* and *C. dubliniensis* were observed to have a preferential capacity to colonize the periodontal pockets.

The presence of these two yeast species and their possible role in the genesis of the chronic periodontal disease and/or the exacerbation of the clinical condition, with respect to the destruction of periodontal tissue, remain unclear topics that should be further studied due to their possible therapeutic implications.

Acknowledgements

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This paper was first published online on iFirst on 25 June 2008.

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