doi: 10.7213/archivesoforalresearch.09.002.AO05 ISSN 2236-8035
Archives of Oral Research, v. 9, n. 2, p. 177-184, May/Aug. 2013

Anaerobic enhancement of protease secretion by periodontal Candida albicans strains

Aumento anaeróbio da secreção de protease pelas cepas de Candida albicans periodontal

Edvaldo Antonio Ribeiro Rosa[a], Alinne Ulbrich Mores Rymovicz[a], Rodrigo Nunes Rached[a], Ana Maria Trindade Grégio[a], Wander José da Silva[b], Lakshman Perera Samaranayake[c]

[a] Xenobiotics Research Unit, Pontifícia Universidade Católica do Paraná. Rua Imaculada Conceição, 1155, Curitiba, 80215-901, PR, Brazil, e-mail: edvaldo.rosa@pucpr.br
[b] Department of Prosthesis and Periodontology, Faculty of Dentistry, Universidade Estadual de Campinas, Piracicaba, SP, Brazil.
[c] Oral Biosciences Unity, Prince Philip Dental Hospital, University of Hong Kong. 5F/34 Hospital Road, Hong Kong.

Abstract

Introduction: Among other non-bacterial organisms, yeasts have been isolated from subgingival sites with relative frequency. *Candida albicans* is the species most commonly isolated although its role in periodontal disease has not been established. **Objective**: This study evaluated the secretion patterns of aspartyl-protease (Sap) by periodontal and nonperiodontal *Candida albicans* strains in normoxic and anoxic conditions. **Material and methods**: Periodontal strains (n=10; periodontal pockets ≥3.00 mm) and nonperiodontal *Candida albicans* strains (n=10) were grown under normoxic and anoxic conditions in protease-inducible broth. Sap activities were quantified in supernatants using azocasein as substrate. Whole-protein contents in supernatants were determined by Bradford's method. Specific protease activities (Sap activity.protein-1) were assessed and compared. **Results**: While nonperiodontal strains secrete similar amounts of Sap under both atmospheric conditions, periodontal strains secrete reduced amounts in the presence of molecular oxygen. **Conclusion**: Despite the limited number of assayed isolates, the possibilities of adaptation or selection of candidal strains to periodontal microenvironment may be considered.

Keywords: *Candida albicans.* Sap. Periodontal.

Resumo

***Introdução**: Entre organismos não bacterianos, as leveduras têm sido isoladas de sítios subgengivais com relativa frequência. Candida albicans é a espécie mais comumente isolada, embora seu papel na doença periodontal não esteja estabelecido. **Objetivo**: Este estudo avaliou os padrões de secreção de aspartil-protease (Sap) por cepas periodontais e não periodontais de Candida albicans em situações de normóxia e anóxia. **Material e métodos**: Cepas periodontais (n=10; bolsas periodontais ≥3,00 milímetros) e cepas de não periodontais (n=10) Candida albicans foram cultivadas sob condições normóxicas e anóxicas em caldo de protease-induzida. A atividade Sap foi quantificada em sobrenadantes utilizando azocaseína como substrato. O conteúdo de proteínas totais nos sobrenadantes foi determinado pelo método de Bradford. Atividades de protease específica (atividade de proteína Sap-1) foram avaliadas e comparadas. **Resultados**: Apesar das cepas não periodontais secretarem quantidades semelhantes de Sap em ambas as condições atmosféricas, as cepas periodontais secretam quantidades reduzidas na presença de oxigênio molecular. **Conclusão**: Apesar do número limitado de amostras analisadas, as possibilidades de adaptação ou seleção de cepas de Candida no microambiente periodontal pode ser considerada.*

***Palavras-chave**: Candida albicans. SAP. Periodonto.*

Introduction

The role of some bacteria in the triggering of periodontal disease is well known. However, some microorganisms belonging to other kingdoms or even domains have received special attention in the last decade (14, 15, 22, 25). Their role in the pathogenesis of periodontal disease still remains uncertain. Among them, *Candida albicans* has been recovered from crevicular sulci and periodontal pocket with a considerable frequency (20, 31).

Candida albicans is a yeast-like organism that may provoke lesions in the oral mucosa that may vary from mild to severe, according to the local/systemic immune status of the host (17). Despite its opportunistic behavior, experimental evidence has pointed toward the existence of a variable display in its virulence attributes. Moreover, growth phenotypes (18) and xenobiotics may modulate such virulence (1, 4, 16, 30).

The family of isoenzymes known as secreted aspartyl proteases (Sap1 to Sap10) is considered as the main putative virulence attribute involved in candidal pathogenesis (19). Saps are responsible for depolymerizing extracellular proteins in order to facilitate the amino acid uptake and the cell–tissue invasion (12). The expression of Saps is dependent on environmental signals (32), and the signal transduction of some isoforms seems to be regulated by a mitogen–activated protein kinase cascade ending in the transcription factor Cph1p and a cyclic AMP–dependent signaling pathway ending in the transcriptional regulator Efg1p (26).

Differences in Sap secretion rates have already been asserted for strains isolated from a variety of sites, but not for strains from periodontal pockets. Recently, our group reported that the lack of molecular oxygen leads candidal strains to secrete more Sap than they could secrete in normoxic conditions (21). To test the hypothesis that anoxia is also important for virulence modulation of periodontal *C. albicans* strains, we carried out the following experiment that evaluated the secretion of Sap in normoxic and anoxic conditions.

Material and methods

***Candida albicans* strains**. Strains 3A1, 13A3, 14A3, 15A2, 30A2, 30A4, 47A4, 55A3, 57A1, and 58A4 were recovered from periodontal pockets with depth equal or higher than 3.00 mm of immunocompetent and non-related patients with chronic periodontitis, and were kindly provided by Professor Jose F. Höfling (Faculty of Dentistry of Piracicaba – Universidade Estadual de Campinas, Brazil). Through RAPD fingerprinting tests, it was confirmed that these strains are not related with each other, being a sub-set of periodontal strains from other study (2). The strains B3, B8, C1, F1,

F2, N1, N2, and N3 were recovered from different cases of mucosal candidosis (29) and are not related to periodontal disease. Strains SC5314 and ATCC®90028™ are reference strains.

Aerobic inocula preparation. Cells of *C. albicans* were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 37 °C. The cultures were monitored until they achieved an OD_{660nm} equal to 0.8. Then, they were harvested, washed three times with sterile 50 mM PBS (pH 6.5), and stored at 4 °C in the same buffer. They were designated as the aerobic cultures of *C. albicans*.

Anaerobic inocula preparation. Aerobic 100 µL inocula (OD_{660nm}=0.8) were used to inoculate tubes with 5 mL of degassed reduced YPD broth (YPD plus 200 µL of 1 mM oleic acid in 100% methanol, 200 µL of 4 mM nicotinic acid, and 10 mg of solid L-cysteine; pH=5.0) that were incubated in an atmosphere with 90% N2, 5% CO2, and 5% H2. The cultures were incubated at 37°C until they achieved an OD_{660nm} equal to 0.8, when 500 µL were used to inoculate another 5 mL of reduced YPD. Again, the cultures were followed until an OD_{660nm} equal to 0.8. Then, they were harvested, washed three times in sterile 50 mM PBS (pH 6.5) plus 0.1% L-cysteine, and anoxically stored at 4 °C in the same buffer. They were designated as the anaerobic cultures of *C. albicans*.

Normoxic production of Saps. Sterile screw-caped polyethylene sample containers containing 30 mL of inducing YNB-BSA broth [yeast nitrogen base (Difco Lab, Inc.), 0.2% filter-sterile BSA fraction V (Sigma-Aldrich, Inc.), and 100 mM glucose (pH 5.0)] received the aerobic inocula (100 µL of cell suspension with OD_{660nm}=0.8) and were incubated in normal atmosphere at 37 °C for 10 d.15

Anoxic production of Saps. Sterile screw-caped polyethylene sample containers containing 30 mL of degassed and reduced YNB-BSA broth (YNB-BSA plus 200 µL of 1 mM oleic acid in 100% methanol, 200 µL of 4 mM nicotinic acid, and 10 mg of solid L-cysteine) received the anaerobic inocula (100 µL of cell suspension with OD_{660nm}=0.8) and were incubated in an anoxic atmosphere with 90% N2, 5% CO2, and 5% H2 at 37 °C, for 10 d.

Quantification of protein. After 10 d of incubation, the aerobic/anaerobic cultures were centrifuged and the supernatants were transferred to sterile clean tubes. The protein concentration of supernatants was determined by absorbance following the method of Bradford, with BSA as standard.

Protease activity. The hydrolyzing activity of the protease was determined using azoalbumin as substrate and the method of Carvalho et al. (4), with minor modifications. Briefly, the supernatants (300 µL) were transferred to glass tubes containing 500 µL of 1% azoalbumin (Sigma Aldrich, Inc.) solution (in 50 mM Tris-HCl buffer, pH 8.0), and the reaction mixtures were incubated for 60 min at 37 °C. The reactions were stopped by adding 800 µL of 10% trichloroacetic acid (TCA) and incubating for further 10 min at room temperature followed by centrifugation at 10,000 rpm for 10 min. Five-hundred microliters of supernatants after TCA precipitation were mixed with equal volumes of 500 mM NaOH and incubated for 15 min. The development of color was colorimetrically measured by taking the absorbance at 440 nm. A control assay, without the supernatant in the reaction mixture, was performed and used as the blank in all measurements. One unit of enzyme activity was defined as the amount of enzyme necessary to increase in 0.001 unit of absorbance at 440 nm per min of digestion. The number of units of activity per milligram of protein was taken as the specific enzymatic activity.

Statistics. All the experimental steps above were repeated in triplicates, in three different moments, obtaining nine individual measures. All the data were evaluated in relation to their homogeneity of variances by the Levene index and analyzed by the Games-Howell and Tukey HDS tests with the statistical package SPSS 15.0 (SPSS Inc., Chicago, Il.). Comparison of enzymatic activity for each *C. albicans* strain was carried out after analysis of normality by Kolmorogov-Smirnov and Shaphiro-Wilk and subsequently by the Kruskal-Wallis test. A p value of 0.05 was assumed as threshold for differences.

Results

Biofilm formation was detected in all aerobic cultures after ten days of incubation. On the other hand, among the twenty strains, only strain SC5341 developed a consistent biofilm on the flasks under anoxic conditions. The remaining strains generated thin and loosen biofilms in the absence of molecular oxygen.

All strains, independent of their origin or atmospheric environment, secreted Saps. The strain-to-strain variations among nonperiodontal strains are presented in Figure 1. Four out of ten strains showed higher enzymatic activity ($p \leq 0.0489$) when grown in normoxic conditions. Three out of ten inverted such behavior secreting more Saps when in anoxia ($p \leq 0.0277$). The differences for the remaining three strains could not be supported by the statistics tests used here ($p \geq 0.1551$).

A more uniform secretion pattern was achieved with periodontal strains (Fig. 2). Excepting strain 58A5, all the others had an increment in the Sap secretion rates with $p \leq 0.0108$ when grown in the absolute absence of molecular oxygen.

Nonparametric multiple comparisons among the four subgroups revealed that in anoxic conditions periodontal strains tend to secret significantly higher ($p < 0.0001$) amounts of Sap than in normoxic conditions (Fig. 3). However, this superior

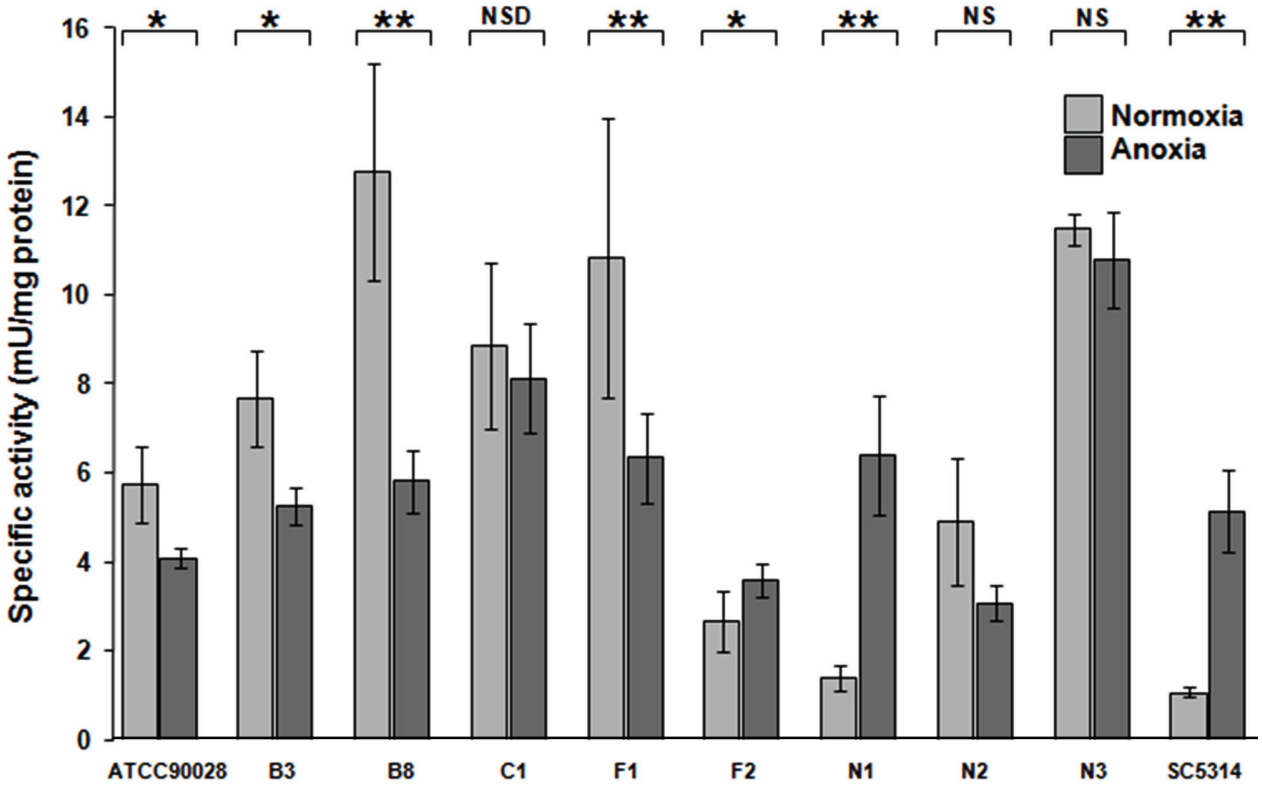

Figure 1 - Comparative of specific activities of non-periodontal C. albicans strains grown in normoxia and anoxia. Each histogram bar represents the mean of nine measures. Error bars represent the standard error of mean; NSD denotes no statistical differences; * denotes differences with $p < 0.05$; ** denotes differences with $p < 0.005$.

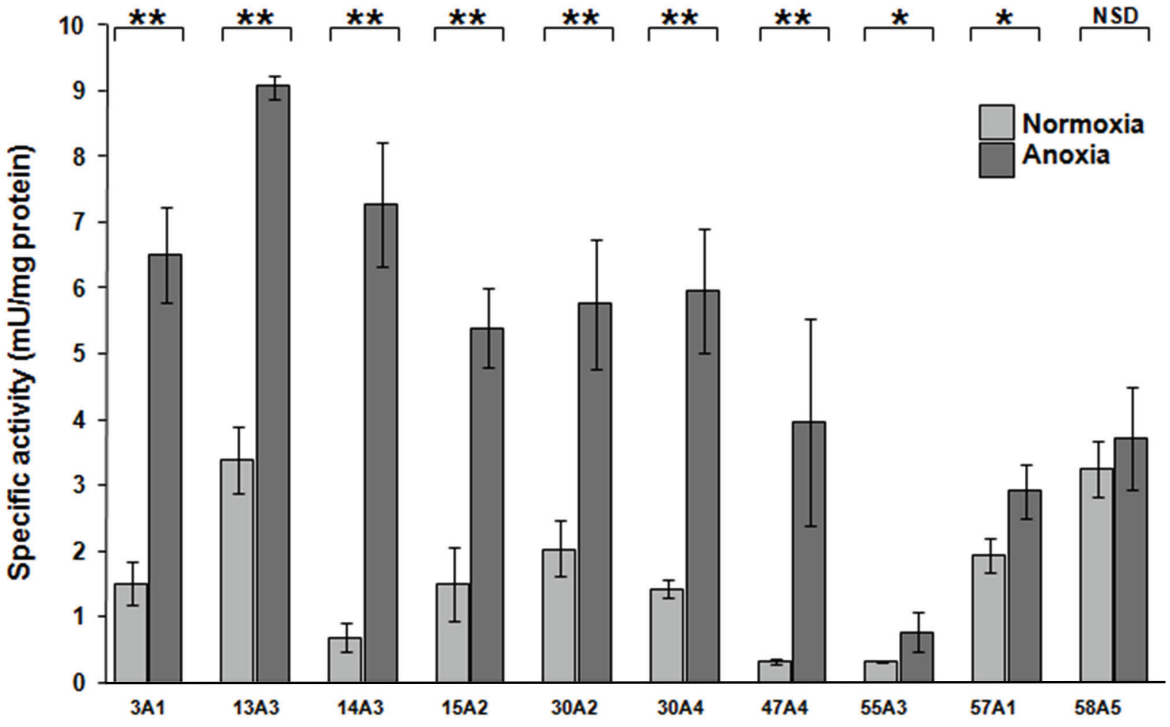

Figure 2 - Comparative of specific activities of periodontal C. albicans strains grown in normoxia and anoxia. Each histogram bar represents the mean of nine measures. Error bars represent the standard error of mean; NSD denotes no statistical differences; * denotes differences with $p < 0.05$; ** denotes differences with $p < 0.005$.

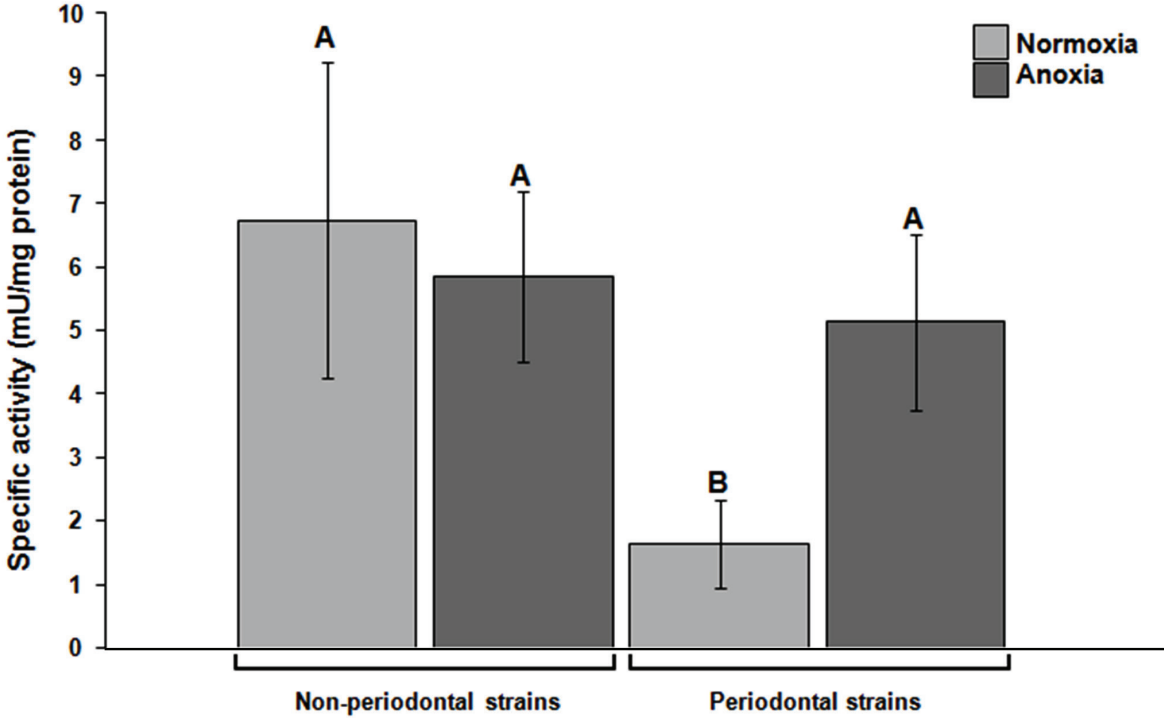

Figure 3 - Comparative mean of specific activities of non-periodontal and periodontal C. albicans strains grown in normoxia and anoxia. Each histogram bar represents the mean of ninety measures. Error bars represent the standard error of mean. Different letters over error bars denote statistical differences.

secretion rate is not different from those obtained for nonperiodontal strains grown in normoxic or anoxic conditions.

Discussion

In Periodontology, the interest on *Candida* spp. just began in the late 1980's and early 1990's (9, 10). However, while in other areas yeasts were considered as primary pathogens (10, 24), in Periodontology they remained left in second plan. In part, it was due to the high involvement of bacteria in the well-reported aggression to periodontal tissues and to the underestimation of fungal pathogenic potential when cells are in anoxic sites. In fact, the influence of anoxia on *Candida* spp. growth and metabolism has received attention only recently (3, 6, 7, 27, 29). Regarding the virulence of periodontal *C. albicans*, few studies have prospected its potential (2, 11), and a wide window of possibilities is open.

The results obtained here point to the possibility of fungal adaptation to the subgingival environment with a proportional higher secretion of proteases in anoxia than in normoxia. Add to this the fact that almost all individual strains repeated such behavior. On the other hand, nonperiodontal strains had a more diverse behavior with no differences for strains grown in normoxia and anoxia. At this point, two possibilities arise. First, there is a pressure exerted by the environmental conditions of periodontal pockets that select candidal strains with similar patterns of protease secretion. This hypothesis is enforced by data from previous studies that propose that few biotypes of *C. albicans* in human periodontal pockets appear to be selected and display closed phenotypes (31) with few clonal types occurring (2). The second idea is that distinct genotypes suffer a similar environmental modulation on virulence attributes when in situations mimicking the pocket conditions. In other words, under absence of molecular oxygen different periodontal strains display similar behavior.

Despite the reduced number of strains analyzed here, we are prone to propose the possibility of intersection between these two hypotheses. We base this premise on two independent points. First, the strains had a RAPD similarity range varying from 0.684 to 0.938 (2). Although not considered as "genetically related isolates (clones)" by the classical Tenover's classification (28), it is plausible that subgingival sites have selected strains with genotypes partially conserved, which is in accordance with the first theory. Second, the fact that periodontal strains had consistent higher specific activity in anoxia (on average and in 90% of the cases) than in normoxia indicates that the second theory also sounds plausible.

Using a semi-quantitative plate assay, our group reported that periodontal strains secrete more Sap when in absence of oxygen (21) and this led us to confirm this fact using a more accurate tool. In that study, nonperiodontal strains also had a similar behavior. The results of both studies may be considered contradictory. As colorimetric assays using chromogenic substrata have superior capacity to assess enzymatic activity than plate methods (8), we believe this matter is solved and the results presented here may be closer to the reality. By comparison, we concluded that anoxia induces Sap secretion similar to nonperiodontal strains isolated from mucosal candidosis cases. Other important point is that both sets of strains, after being isolated from patients, were conserved in laboratory collections with constant replatings. As the virulence patterns were markedly different for periodontal strains in distinct atmospheres, we believe that they did not suffer any domestication, with generalized loss in virulence. Domestication is a common phenomenon involving alterations in candidal genotype during strains maintenance in laboratories over long periods of time (13). Based on this, we hypothesize that this may be due to the constitutive behavior of SAP genes (5) and anoxia-related control of expression by the transcription factor Efg1 (23). As the domestication phenomena require long periods of time to occur, the "nondomesticated" behavior may be a direct reflex of the selection/adaptation possibilities we have pointed out above for periodontium-related strains.

Regarding the fact that strain SC5314 generated thicker anaerobic biofilm than others, such finding is somehow surprising, since it was expected that all strains could form consistent biofilms (29). However, when the anaerobic specific activities for all strains are compared, it is perceptible that such increment in the thickness of strain SC5314 biofilm does not reflect in virulence increasing. Further experiments involving strictly candidal biofilms (18)

might be useful to complement the present study to respond if cell loads imply more virulent biofilms.

Conclusion

Within the limits of this study, the results obtained allow us to infer that the proteolytic behavior of periodontium-related *C. albicans* strains is dependent on the absence of molecular oxygen.

Acknowledgements

The authors are in debt with Professor José F. Höfling (Unicamp) and Dr. Zaw M. Thein (HKU) for the yeast strains, with Ms Joyce Yau and Ms Becky Cheung (HKU) for their technical support, and with Professor Sérgio A. Ignácio (PUCPR) for statistics analyses. This study was supported by grants supplied by The Araucaria Foundation (Process 9042), CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Process BEX 0559/06-7), and intramural funds from Oral Biosciences Unit-HKU.

References

1. Baboni FB, Barp D, de Azevedo-Izidoro AC, Samaranayake LP, Rosa EA. Enhancement of Candida albicans virulence after exposition to cigarette mainstream smoke. Mycopathologia. 2009;168:227-235.
2. Barros LM, Boriollo MF, Alves AC, Klein MI, Gonçalves RB, Höfling JF. Genetic diversity and exoenzyme activities of Candida albicans and Candida dubliniensis isolated from the oral cavity of Brazilian periodontal patients. Arch Oral Biol. 2008;53:1172-1178.
3. Biswas SK, Chaffin WL. Anaerobic growth of Candida albicans does not support biofilm formation under similar conditions used for aerobic biofilm. Curr Microbiol. 2005;51:100-104.
4. Carvalho AP, Gursky LC, Rosa RT, Rymovicz AU, Campelo PM, Grégio AM, Koga-Ito CY, Samaranayake LP, Rosa EA. Non-steroidal anti-inflammatory drugs may modulate the protease activity of Candida albicans Microb Pathog. 2010;49:315-322.
5. Dubois N, Colina AR, Aumont F, Belhumeur P, de Repentigny L. Overexpression of Candida albicans secretory aspartyl proteinase 2 and its expression in Saccharomyces cerevisiae do not augment virulence in mice. Microbiology. 1998;144:2299-2310.
6. Dumitru R, Navarathna DH, Semighini CP, Elowsky CG, Dumitru RV, Dignard D, Whiteway M, Atkin AL, Nickerson KW. In vivo and in vitro anaerobic mating in Candida albicans. Eukaryot Cell. 2007;6:465-472.
7. Ernst JF, Tielker D Responses to hypoxia in fungal pathogens. Cell Microbiol. 2009;11:183-190.
8. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev. 2000;13:122-143.
9. González S, Lobos I, Guajardo A, Celis A, Zemelman R, Smith CT, Saglie FR. Yeasts in juvenile periodontitis Preliminary observations by scanning electron microscopy. J Periodontol. 1987;58:119-124.
10. Jacobsen MD, Duncan AD, Bain J, Johnson EM, Naglik JR, Shaw DJ, Gow NA, Odds FC. Mixed Candida albicans strain populations in colonized and infected mucosal tissues. FEMS Yeast Res. 2008;8:1334-1338.
11. Järvensivu A, Hietanen J, Rautemaa R, Sorsa T, Richardson M. Candida yeasts in chronic periodontitis tissues and subgingival microbial biofilms in vivo. Oral Dis. 2004;10:106-112.
12. Koelsch G, Tang J, Loy JA, Monod M, Jackson K, Foundling SI, Lin X. Enzymic characteristics of secreted aspartic proteases of Candida albicans. Biochim Biophys Acta. 2000;1480:117-131.
13. Lehmann PF, Wu LC, Mackenzie DW. Isoenzyme changes in Candida albicans during domestication. J Clin Microbiol. 1991;29:2623-2635.
14. Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA. Methanogenic Archaea and human periodontal disease. Proc Natl Acad Sci USA. 2004;101:6176-6181.

15. Li CL, Liu DL, Jiang YT, Zhou YB, Zhang MZ, Jiang W, Liu B, Liang JP Prevalence and molecular diversity of Archaea in subgingival pockets of periodontitis patients. Oral Microbiol Immunol. 2009;24:343-346.

16. McCool L, Mai H, Essmann M, Larsen B Tetracycline effects on Candida albicans virulence factors. Infect Dis Obstet Gynecol. 2008:493-508.

17. McCullough MJ, Ross BC, Reade PC. Candida albicans: a review of its history, taxonomy, epidemiology, virulence attributes, and methods of strain differentiation. Int J Oral Maxillofac Surg. 1996;25:136-144.

18. Mendes A, Mores AU, Carvalho AP, Rosa RT, Samaranayake LP, Rosa EA. Candida albicans biofilms produce more secreted aspartyl protease than the planktonic cells. Biol Pharm Bull. 2007;30:1813-1815.

19. Naglik JR, Challacombe SJ, Hube B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev. 2003;67:400-428.

20. Rams TE, Slots J. Candida biotypes in human adult periodontitis. Oral Microbiol Immunol. 1991;6:191-192.

21. Rosa EA, Rached RN, Ignácio SA, Rosa RT, José da Silva W, Yau JY, Samaranayake LP. Phenotypic evaluation of the effect of anaerobiosis on some virulence attributes of Candida albicans. J Med Microbiol. 2008; 57:1277-1281.

22. Saygun I, Kubar A, Sahin S, Sener K, Slots J. Quantitative analysis of association between herpes viruses and bacterial pathogens in periodontitis. J Periodontal Res. 2008;43:352-359.

23. Setiadi ER, Doedt T, Cottier F, Noffz C, Ernst JF. Transcriptional response of Candida albicans to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. J Mol Biol. 2006;361:399-411.

24. Siqueira-Jr JF, Sen BH. Fungi in endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97:632-641.

25. Slots J. Herpes viral-bacterial synergy in the pathogenesis of human periodontitis. Curr Opin Infect Dis 2007;20:278-283.

26. Staib P, Kretschmar M, Nichterlein T, Hof H, Morschhäuser J. Transcriptional regulators Cph1p and Efg1p mediate activation of the Candida albicans virulence gene SAP5 during infection. Infect Immun. 2002;70:921-927.

27. Stichternoth C, Ernst JF. Hypoxic adaptation by Efg1 regulates biofilm formation by Candida albicans. Appl Environ Microbiol. 2009;75:3663-3672.

28. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233-2239.

29. Thein ZM, Samaranayake YH, Samaranayake LP. In vitro biofilm formation of Candida albicans and non-albicans Candida species under dynamic and anaerobic conditions. Arch Oral Biol. 2007;52:761-767.

30. Ueta E, Tanida T, Yoneda K, Yamamoto T, Osaki T. Increase of Candida cell virulence by anticancer drugs and irradiation. Oral Microbiol Immunol. 2001;16:243-249.

31. Urzúa B, Hermosilla G, Gamonal J, Morales-Bozo I, Canals M, Barahona S, Cóccola C, Cifuentes V. Yeast diversity in the oral microbiota of subjects with periodontitis: Candida albicans and Candida dubliniensis colonize the periodontal pockets. Med Mycol. 2008;46:783-793.

32. White TC, Agabian N. Candida albicans secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J Bacteriol. 1995;77:5215-5221.

Received: 10/26/2013
Recebido: 26/10/2013

Accepted: 12/09/2013
Aceito: 09/12/2013