

Quantification and Pathogenicity of *Candida albicans* in Denture-Wearing and Nondenture-Wearing Elderly

Boy M. Bachtar¹ Turmidzi Fath² Retno Widowati³ Endang W. Bachtar¹

¹Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia

²Department of Biology, Graduate School, Universitas Nasional, South Jakarta, Indonesia

³Department of Biology, Graduate School, Universitas Nasional, South Jakarta, Indonesia

Address for correspondence Endang W. Bachtar, M.Biomed, Ph.D, Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, JL. Salemba Raya 4, Jakarta 10430, Indonesia (e-mail: endang04@ui.ac.id).

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Abstract

Objective The primary purpose of this study was to evaluate and compare the microbial loads and pathogenicity traits of oral *Candida albicans* in denture-wearing (DW; $n = 15$) and nondenture-wearing (NDW; $n = 15$) elderly persons.

Materials and Methods The fungal counts of the saliva, tongue dorsa, and prosthesis-fitting surfaces of the participants were assessed using real-time polymerase chain reaction to compare the quantity and expression of selected *C. albicans* biofilm-associated genes (*ALS3*, *HWP1*, and *YWP1*).

Statistical Analysis The obtained data were analyzed by one-way analysis of variance, followed by Bartlett's test. When appropriate, the Student's *t*-test was also used; a value of $p < 0.05$ was considered statistically significant.

Results In both groups, the count of *C. albicans* was found to be significantly higher in saliva than in other oral samples. The expression of the hypha-specific genes (*ALS3* and *HWP1*) in the tongue dorsa was higher in the DW group ($p < 0.05$), whereas the transcription level of the yeast-specific gene (*YWP1*) was significantly higher in the NDW group.

Conclusion Both tongue dorsa and dentures appear to be sharing factors that are important for *C. albicans* biofilm growth in abiotic and biotic oral surfaces of the elderly.

Keywords

- *Candida albicans*
- tongue
- denture
- *ALS3*
- *HWP1*
- *YWP1*

Introduction

Candida albicans is a unique opportunistic fungal species that, together with other oral microflora, can exist in biofilms, on either host tissues or abiotic surfaces in humans.^{1,2} Since biofilm formation is a fungal virulence trait, *C. albicans* colonization and biofilm formation on oral mucosa, tooth surfaces, and dental prostheses are of clinical significance. From a prosthetic oral rehabilitation perspective, denture-wearing (DW) should be considered a predisposing factor for oral candidiasis.³ Therefore, knowledge of pathologies related to prosthetic microbial plaque is essential for maintaining oral health, particularly because oral physiological changes such as impaired salivary flow commonly occur in the elderly. Subsequent to these changes, *C. albicans* grows

in hypha form due to the lack of a salivary component called statherin, which inhibits hyphal growth by inducing the transition of hyphae to lateral yeast cells.⁴ In this context, following our previous study on detecting *C. albicans* in children with early childhood caries experience,⁵ we aimed to further investigate the characteristics of *C. albicans* pathogenicity in elderly subjects. Therefore, we determined the extent to which *C. albicans* pathogenicity is affected by denture insertion in elderly people. We used real-time polymerase chain reaction (PCR) to assess the fungal load and the expression of selected biofilm-associated genes (*ALS3*, *HWP1*, and *YWP1*). We analyzed whether the genes were transcribed at similar levels in different oral niches (tongue dorsa and prosthesis-fitting surfaces) of DW and nondenture-wearing (NDW) elders.

Materials and Methods

Participants

Thirty elderly adults, aged ≥ 60 years (male and female), were randomly selected for this study. They were living in two geriatric homes located in a rural area of Jakarta, the capital city of Indonesia. Participants who had illnesses or medical problems (systemic diseases, pharmacological therapies, etc.) were not included in the study.

Samples of their saliva, dentures, and tongue dorsa were obtained (**►Fig. 1**). The participants were categorized into two groups: denture wearers ($n = 15$), with maxillary or mandibular dentures (partial or full), and nondenture wearers ($n = 15$).^{6,7}

Other inclusion criteria were no smoking, no fasting, and no use of antibiotics for 3 months before this study. The participants were instructed not to eat and/or drink for at least 2 hours before the oral sample collection.

The samples were collected, after informed consent was obtained from all the participants, in accordance with the approved protocol of the Bioethics Committee of the Faculty of Dentistry, Universitas Indonesia (protocol number 020950818). The protocol conformed to the criteria of the Helsinki Declaration and the good clinical practical guidelines of the International Council on Harmonization.

Unstimulated saliva (minimum volume of 2 mL) was collected by spitting into a sterile Falcon tube. The other oral samples (from dentures and tongue dorsal surfaces) were collected using a sterile cotton swab. Samples from dentures were collected by swabbing the mucosal surface five times; for those from the tongue dorsa, the swabbing was done vertically (five times), from the circumvallate papillae to the tip of the tongue. All the collected samples were then placed into an Eppendorf tube and immediately cold-transported to the laboratory, where the samples were stored at -80°C until further use. For our analysis, we focused on the quantity of *C. albicans* and the mRNA expression of selected biofilm-associated genes (*ALS3*, *HWPI*, and *YWP1*) in the tongue surfaces, denture-fitting surfaces, and saliva. We also analyzed

the association between the *C. albicans* count and the transcription level of each gene tested.

Quantification of *C. albicans* by qPCR

The method used to extract fungal genomic DNA from each sample was the same as that reported previously using the Trizol reagent (Sigma-Aldrich; Dorset, UK).⁵ The concentration and quality of the obtained DNA were determined using Qubit assay reagents (Invitrogen; Carlsbad, California, United States); after dissolving in Tris-EDTA buffer, the DNA was cooled to -20°C for further analysis. Then, the DNA samples were quantified through quantitative PCR (qPCR) with the *C. albicans*-specific primers²² shown in **►Table 1**. For PCR quantification, each sample was run in triplicate on an ABI StepOnePlus Real-Time PCR System, wherein an SYBR Green PCR Master Mix (Applied Biosystems) was used in accordance with the protocol provided by the company.

The PCR conditions were a final reaction volume of 10 μL (composed of 50 ng of sample DNA and 1 μM of primer) and thermal cycling conditions of a 10-minute initial denaturation at 95°C , followed by 40 cycles of denaturation at 95°C for 15-second annealing at 60°C for 60 second and elongation at 95°C for 15 seconds. The qPCR product was visualized as a melting curve (95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds) and the cycle threshold (C_t) value was converted to log colony forming unit (CFU).

The amount of genomic DNA of *C. albicans* was estimated by constructing a standard curve with r^2 , as shown in **►Fig. 2A**. Briefly, we performed a 10-fold serial dilution of extracted *C. albicans* (ATCC 10231). The number of viable fungal cells (CFU/mL) was assessed by plating culture dilutions on Sabouraud dextrose agar, and the same strain was used as a positive control while running the qPCR. Therefore, the amount of *C. albicans* in the oral samples was determined by plotting the C_t values against the log of the standard curve, whereas the melting peaks were used to assess the specificity of the amplicon (**►Fig. 2B–D**).

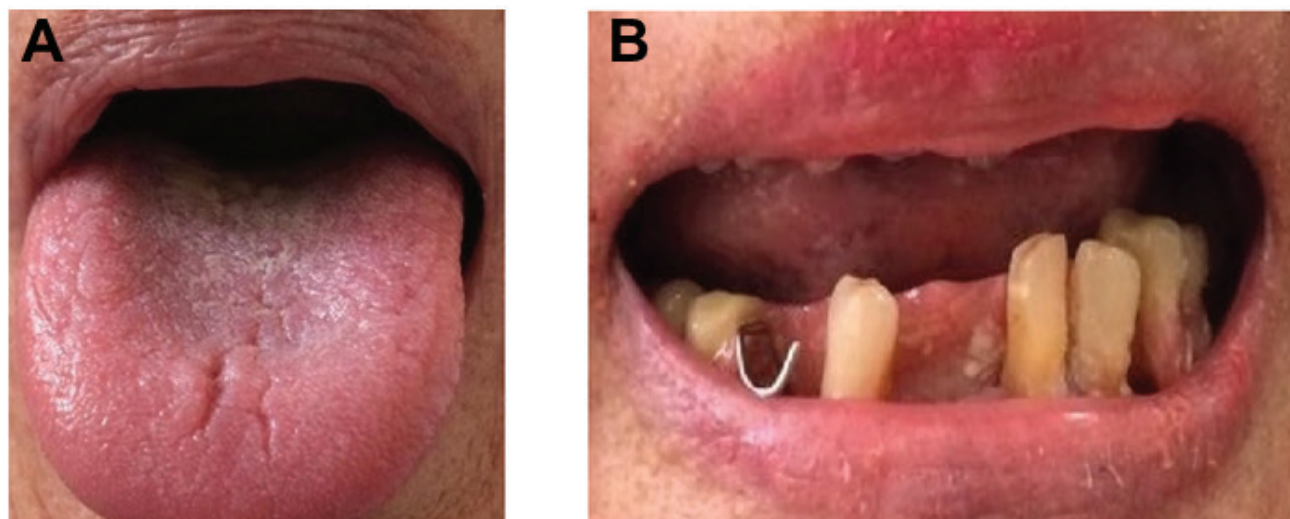
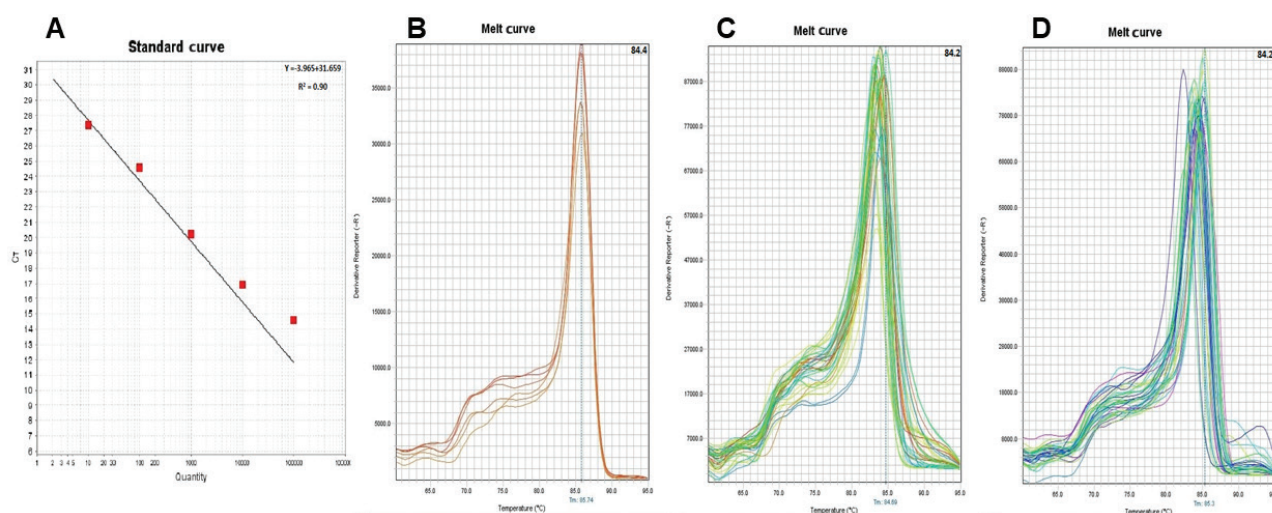


Fig. 1 Sample profiles of elderly subjects. Tongue dorsa (A) and dentures (B).

Table 1 Primers used in this study

Primer	Sequences	Reference
ACT1	F: 5'-TTTCATCTTCTGTATCAGAGGAAGTATTT-3'	8
	R: 5'-ATGGGATGAATCATCAACAAGAG-3'	
18S rRNA	F: 5'-CACGACGGAGTTTCACAA GA-3'	22
	R: 5'-CGA TGG AAG TTT GAG GCA AT-3'	
YWP1	F: 5'-GCTACTGCTACTGGTGCTA-3'	22
	R: 5'-AACGGTGGTTTCTTGAC-3'	
HWP1	F: 5'-GCTCCTGCTCCTGAAATGAC-3'	22
	R: 5'-CTGGAGCAATTGGTGAGGTT-3'	
ALS3	F: 5'-CAACTGGGTTATTGAAACAAAAACA-3'	22
	R: 5'-AGAAACAGAAACCAAGAACAACC-3'	

**Fig. 2** Standard curves and melt curves. Standard curve of *Candida albicans* ATCC 10231 (A) and representative melt curve graphs for *C. albicans* count in saliva (B), tongue dorsum (C), and denture mucosal surface (D).

qPCR Analysis for mRNA Expression of *C. albicans* ALS3, HWP1, and YWP1 in Tongue Dorsa and Denture Samples

RNA isolation, purification, and reverse transcription of cDNA were performed similar to the method used in a previous study.⁵ Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Life Technologies, Carlsbad, California, United States), a passive reference (ROX, Invitrogen), and primers for each gene tested (►Table 1), as well as 1 µg of cDNA, were used to quantify the cDNA. The qPCR cycling conditions consisted of a 10-minute initial denaturation at 95°C followed by 40 PCR cycles of 15 seconds at 95°C and 1 minute at 60°C. The formula of fold change $2^{-\Delta\Delta Ct}$ was used to calculate the relative mRNA expression, which was compared with that of the housekeeping gene, *ACT1*⁸; the mRNAs of genes (*ALS3*, *HWP1*, and *YWP1*) expressed in the saliva samples were used as controls.

Statistical Analysis

All means are reported as mean ± standard error. Statistical comparison between the three oral samples tested was done

using ordinary one-way analysis of variance, followed by the Bartlett's test. The Mann-Whitney U test was used to compare the saliva and tongue dorsa samples of the two groups. Differences were considered significant if *p*-value was less than 0.05. The data were analyzed using Prism version 6.0 (GraphPad Software, Inc.; San Diego, California, United States).

Result

Candida albicans was present in all the samples collected from both the groups of participants. In general, although interindividual variation was observed in both groups, the count of *C. albicans* in saliva samples was higher than that in other oral samples (►Fig. 3A and 3B) (*p* < 0.05). Moreover, the amount (log DNA copies) of *C. albicans* in the saliva of the DW group was significantly higher than that of the NDW group (*p* < 0.05). Lastly, although the tongue dorsa samples of subjects in the NDW group showed, on average, a slightly higher count of *C. albicans* than that of the DW group, this difference was not statistically significant.

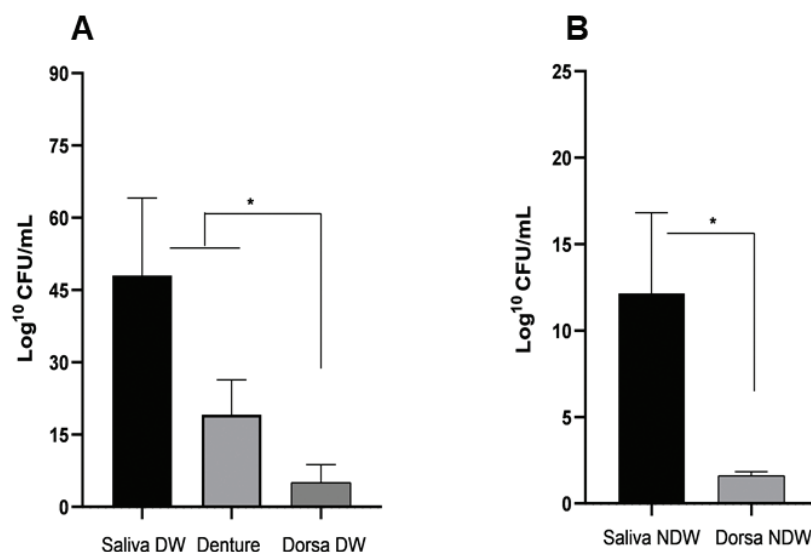


Fig. 3 Standard error of absolute numbers of *Candida albicans* detected in oral samples (saliva, dentures, and tongue dorsa) of denture-wearing (A) and nondenture-wearing (B) elderly subjects. * $p < 0.05$. DW, denture wearing; NDW, nondenture wearing.

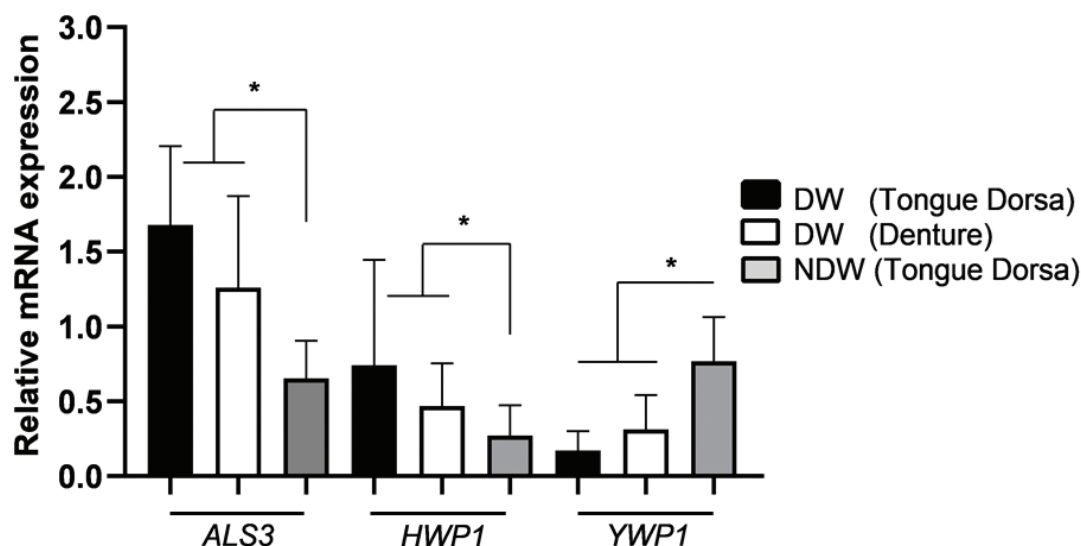


Fig. 4 mRNA expression of selected biofilm-associated genes (*ALS3*, *HWP1*, and *YWP1*). Fold changes in gene expression on tongue dorsa of denture-wearing (A) and nondenture-wearing (B) elders were compared. All quantitative polymerase chain reaction values were normalized according to the expression of the housekeeping gene *ACT1*. Error bars show standard error from triplicate determinations. * $p < 0.05$. DW, denture-wearing; NDW, nondenture-wearing.

Transcription Levels of *C. albicans* *ALS3*, *HWP1*, and *YWP1*

We further analyzed the mRNA expression of selected *C. albicans* genes (*ALS3*, *HWP1*, and *YWP1*, which are involved in the regulation of the fungus phenotypic transition⁹) in the tongue dorsa of the DW and NDW subjects. The transcription levels of both *ALS3* and *HWP1* were significantly higher in the tongue dorsa of the DW subjects than in the NDW subjects. In contrast, a significantly higher level of *YWP1* mRNA expression was detected in the tongue dorsa of the NDW subjects compared with that in the DW subjects (► **Fig. 4**).

Discussion

The specific aim of this study was to compare the load and pathogenic traits of oral *C. albicans* in DW and NDW elderly persons. In both groups, saliva is the oral niche that has the highest count of *C. albicans*. Hence, although saliva plays a key role in modulating fungal colonization in the oral cavity,¹⁰ the results of this study show that the overall activity of salivary secretion toward *C. albicans* is less effective in older people. This may indicate age-induced changes in the normal protective functions of saliva (cleansing, lubrication, and antibacterial activity).

A study by Leung et al¹¹ showed that low salivary flow rate (SFR) in the elderly contributes significantly to *C. albicans* colonization in the oral cavity. Therefore, denture wearers are more at risk of contracting oral candidiasis.¹² In this study, we did not measure the SFR. However, the high count of salivary *C. albicans* we observed does not indicate that a low SFR has a significant effect on salivary colonization by this fungus, a phenomenon that has been reported previously.¹³ Hence, it is possible that our elderly subjects may have produced saliva containing complementary receptors for *C. albicans* attachment on the inner surface of the dentures. This attachment may involve salivary pellicles (e.g., proline-rich proteins) that act as complementary receptors to which their ligands, known as cell surface molecule adhesins (e.g., mannoproteins and fibrillar adhesins), bind.^{14,15}

Since saliva has been the preferred oral sample in epidemiological studies,¹⁶ we analyzed the difference in the amount of *C. albicans* in saliva collected from the DW and NDW groups. First, we found that there was a significantly higher *C. albicans* load in the saliva samples from both the DW and NDW groups, compared with that in the other oral samples. We also observed that the *C. albicans* count was highest in the saliva of the DW group. We hypothesize that the presence of dentures in DW subjects provides environmental conditions, particularly beneath the denture base, that may favor yeast colonization in the oral cavity. Therefore, both the tongue dorsa and the fitting surfaces of dental prostheses must be considered as important reservoirs of *C. albicans* colonization, from which this microorganism dislodges into saliva. Moreover, the results of this study showed that the *C. albicans* counts on the tongue surfaces of the DW and NDW groups are similar. This may indicate that the oral conditions resulting from DW do not affect the colonization status of the fungus on biotic surfaces (tongue dorsa). Interestingly, within the DW group, *C. albicans* shows a lower affinity for adherence to the tongue dorsa surfaces than to the mucosa-fitting surfaces of the dentures. This result and those of other studies^{17,18} suggest that in elderly subjects, a nonshedding abiotic substratum is an ideal oral habitat for microbial attachment and growth.

Since oral candidiasis occurs on the oral mucosal surface,¹⁹ we further compared the pathogenicity of *C. albicans* in tongue dorsa by analyzing the expression of selected biofilm-associated genes in DW and NDW persons. We chose the *ALS3*, *HWP1*, and *YWP1* genes because the *C. albicans* load in tongue dorsa is associated with an alteration in fungal morphology (from yeast to hyphal form), and upregulation or downregulation of the hypha-specific gene (*HWP1*), adhesion-related gene (*ALS3*),²⁰ and gene involved in the antiadhesive activity of the fungus (yeast-associated gene, *YWP1*).²¹

The transcription levels of *ALS3* and *HWP1* were higher in the DW group than those in the NDW group. Interestingly, *YWP1* was higher expressed in the tongue dorsa of the NDW group than in the DW group. Although a similar overall

carriage rate of *C. albicans* was found in the tongue dorsa of both the groups, the difference in the expression of the selected biofilm-associated genes did not affect the colonization status of the fungus. The dissimilarities between the DW and NDW groups in the expression of both hypha and yeast-associated genes suggested that the NDW subjects harbored stable yeast cells as biofilm components on their tongue dorsa surfaces. Since yeast cells play an important role in the commensalisms and pathogenesis of *C. albicans*,²¹ the tongue was the primary reservoir for *C. albicans* for the NDW subjects that participated in this study.

Conclusions

This preliminary study revealed the difference in the pathogenicity of *C. albicans* (evaluated by qPCR) in DW and NDW elderly persons. The results showed that whole saliva is a better oral sample for detecting *C. albicans* in elderly persons, irrespective of wearing dental prostheses. Furthermore, the adhesion of *C. albicans* to the biotic surfaces of oral cavities is affected by the capacity of the fungus to colonize the inner surfaces of dentures, which ultimately improves its ability to form biofilms on tongue dorsa and become pathogenic in oral environments. These preliminary data, therefore, provide a justification and basis for further exploration of other oral fungal colonization phenomena, particularly, in broader groups of elderly subjects.

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Conflict of Interest

None declared.

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