Influence of nicotine on the adherence of *Candida albicans* ATCC 14053 and *Candida parapsilosis* ATCC 22019 and expression of selected binding-related genes

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**ABSTRACT**

Smoking is considered one of the main factors for development of dental plaque. Yeasts have been described to contribute to the plaque community. The aim of this study was to investigate the influence of nicotine on the growth of planktonic cells and biofilm, cell aggregation, surface hydrophobicity, cell adherence and binding-related genes expressed by *Candida albicans* and *Candida parapsilosis* in vitro. The relative number of viable fungal cells was determined based on viable plate counts. The biofilm growth was quantified using the crystal violet assay. Reverse transcription-polymerase chain reaction was used to evaluate the regulation of selected genes associated with adherence. The results indicated that nicotine enhanced the growth of both planktonic and biofilm oral fungal cells. Cell surface hydrophobicity and the expression of Hyphal Wall Protein 1 (HWP1) and agglutinin-like sequences 3 (ALS3) of *C. albicans* and *C. parapsilosis* were found to increase relative to the nicotine concentrations. The results suggest that nicotine can enhance the growth of *C. albicans* and *C. parapsilosis* in vitro and influence their adherence to the surface of microplate wells that mimic as the tooth surface.

**Introduction**

Cigarette smoke contains more than 4000 types of chemicals, at least 50 of them being very carcinogenic. Approximately 6%–30% of the cigarette content is constituted by nicotine, an alkaloid in cigarette smoke. A stick of cigarette contains 9–30 mg of nicotine; however, the nicotine absorbed in the body by inhalation through smoking is about 0.5–2 mg per cigarette [1]. Although this amount is low, it is sufficient to cause many serious illnesses (e.g. cancer), heart problems and abortion as well as other health problems. Nicotine has been reported to be able to alter certain cellular functions, such as cell growth, attachment, and matrix protein synthesis of oral microbes [2]. Nicotine can also stimulate oral biofilm formation and influence cell metabolism of biofilm microorganisms. Unfortunately, there are still questions about how nicotine increases the metabolic activity of microorganisms in the oral cavity [3].

It has been reported that *Candida* species are more prevalent in tobacco smokers than in non-smokers [4]. The *Candida* genus is comprised of about 150 species of yeast-like fungi. In general, they are classified as asexual diploid fungi that can exist in the form of yeasts or hyphae [5]. *Candida* species such as *C. albicans*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. dubliniensis*, *C. kefyr*, *C. guilliermondii*, *C. parapsilosis* and *C. lipolytica* are present in the oral cavity as commensal microorganisms and these can act as opportunistic pathogens in cases of low immune system [6]. The most commonly isolated species from oral cavities is *C. albicans*, in up to 60% in the general population [7]. In addition, *C. albicans* and *Candida parapsilosis* are frequently reported to be present in oral biofilm [8]. Incubation temperature is one of the factors influencing the growth of *Candida* sp. from normal microbial flora to pathogenic state in the oral cavity. Most studies indicate that the optimal temperature for *C. parapsilosis* growth is 35 °C instead of 37 °C [9]. Specific adhesion between *Candida* and receptors on host tissues results in a stronger covalent attachment in oral biofilm [6]. It is linked with enhanced expression of some virulence genes, HWP1 (Hyphal Wall Protein 1) [10] and ALS (agglutinin-like sequence) [11] which promotes the adhesion of *Candida* species to epithelial surfaces. In addition, interaction between *Candida* species has also been reported to favour biofilm formation in the oral cavity [6].

Thus, the aim of this study was to investigate the effect of nicotine on *C. albicans* and *C. parapsilosis*
planktonic cell growth, biofilm mass, aggregation, surface hydrophobicity, cell adherence and binding-related gene expression.

Materials and methods

Microbial strains, growth condition and chemicals

*C. albicans* ATCC 14053 and *C. parapsilosis* ATCC 22019 were stored in 20% glycerol at −80 °C and thawed at 25 °C before use. The *Candida* cells were then revived in Yeast Peptone Dextrose (YPD) broth (BD Difco™) followed by overnight incubation at 37 °C (35 °C for *C. parapsilosis*). The turbidity of cell suspensions was adjusted and standardized spectrophotometrically to an optical density at 550 nm (OD_{550}) of 0.144, which is equivalent to 1 × 10^6 cells/mL.

Nicotine (>99% (GC), liquid) was purchased from Merck Millipore (Darmstadt, Germany). Throughout the experiment, four different concentrations of nicotine (1, 2, 4 and 8 mg/mL) were used.

Preparation of Candida planktonic cell suspension

A loopful of colonies of each strain were inoculated into YPD broth containing different nicotine concentrations (1, 2, 4 and 8 mg/mL) and incubated overnight at 37 °C (35 °C for *C. parapsilosis*). The cells were harvested by centrifugation at 12,000 g (Jouan A14, Saint Nazaire, France). Then, the cell pellet was washed twice with phosphate-buffered saline (PBS, pH 7.2; Sigma-Aldrich, Saint Louis, USA) and re-suspended in the same buffer. Finally, the turbidity of the cell suspension was adjusted and standardized spectrophotometrically to an OD_{550} of 0.144, which is equivalent to 1 × 10^6 cells/mL.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined according to the Clinical and Laboratory Standards Institute (CLSI) modified method M27-A3 [12]. MIC was considered the lowest concentration that showed absence of growth and MFC, the lowest concentration of nicotine that kills the candidal cells. A 100 μL of YPD broth was dispensed into a microtiter plate (Thermo Scientific™ Nunc™, New York, USA) labelled as Well 1 (W1) to Well 8 (W8). Then, 100 μL of nicotine (16 mg/mL) was added into W3 and two-fold serial dilutions were made from W3 to W8. Hence, the final concentration of nicotine in W3, W4, W5, W6, W7 and W8 was 8, 4, 2, 1, 0.5 and 0.25 mg/mL, respectively. Then, 20 μL of candidal suspension was added to W1 through W8 except W2. Well 1, which contained YPD broth and *Candida* suspension, served as a negative control. The microtiter plates were incubated overnight at 37 °C, except for *C. parapsilosis*, which was incubated at 35 °C. Following incubation, the growth inhibition in the microtiter wells was measured based on OD_{550} nm using a microplate reader (Multiskan EX, Thermo Electron Corp., San Diego, CA, USA).

Minimum biofilm inhibitory concentration (MBIC)

MBIC is defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms. The biomass of *Candida* species was determined by measuring the colour intensity of crystal violet in the microplate wells. Briefly, the candidal cells were cultured in YPD broth and incubated overnight at 37 °C. The cultures were then harvested by centrifugation at 12,000 g and rinsed with PBS. The washed candidal cells were then resuspended in YPD broth and adjusted spectrophotometrically to OD_{550} of 0.144, which represented approximately 1 × 10^6 cells/mL. Then, 20 μL of candidal cultures (1 × 10^6 cells/mL) were respectively added to the microtiter plate wells pre-loaded with different nicotine concentrations (0.25–8.0 mg/mL). A negative control *Candida* group without exposure to nicotine was also incubated in parallel. After 24 h of incubation at 37 °C, the suspension was removed from each well. The biofilm was gently washed twice with PBS and fixed with 10% formaldehyde for 10 min. The plates were washed twice with PBS and stained with 0.5% crystal violet for 1 h followed by washing three times with PBS. In addition, 200 μL of 2-propanol was added to the wells with biofilm layer and incubated for 1 h. Finally, the absorbance was measured using a spectrophotometer at 490 nm with isopropanol as a blank sample.

Growth profile determination of Candida species

To determine the growth curve of candidal cells under the influence of nicotine, 5 mL of *Candida* suspension (1 × 10^6 cells/mL) was added to 45 mL of YPD broth containing various nicotine concentrations (1, 2, 4 and 8 mg/mL) in sterile conical flasks. As a negative control, 45 mL of YPD broth without nicotine was inoculated with 5 mL of *Candida* suspension in a sterile flask. The flasks were placed in a shaking water bath incubator with continuous agitation at 35–37 °C. One milliliter of cell suspension was taken for turbidity measurement periodically, at 1-h intervals over a period of 18 h, using a spectrophotometer at 550 nm. The growth of each species was evaluated based on viable cell counts
(colony-forming units, CFU) which were scored at 2, 6, 10, 14 and 18 h of incubation. The candidal cell suspensions were diluted by serial dilutions in PBS before plating. The OD readings were plotted against time, and the specific growth rate (μ) and doubling time (g) were calculated using the formulas below [13].

(i) Specific growth rate, \( \mu = \ln(N_t/N_0)/(t_2 - t_1) \),

(ii) Doubling time, \( g = \log_{10}(N_t/N_0)/\log_{10}2 \),

where \( N_t \) is the number of cells at log phase; \( N_0 \) is the number of cells at time \( t = 0 \), \( t_2 \) is the time taken to reach a plateau and \( t_1 \) is time, \( t = 0 \) when the cells enter the log phase.

**Cell surface hydrophobicity (CSH) determination**

The non-specific adhesion of oral *Candida* in the presence of nicotine was evaluated by exposing 2 mL of candidal suspension (1 x 10^7 cells/mL) to 1, 2, 4, and 8 mg/mL nicotine for 1 h. The treated cells were then added to 85% saline and the absorbance at 550 nm was taken using a spectrophotometer. The absorbance of each tube represents the initial absorption in the absence of hexadecane (A0). Hexadecane was used to mimic the hydrophobic surfaces of teeth in this study [14]. Thereafter, 200 μL of hexadecane were added to each respective tube and the samples were vortexed vigorously. The tubes were left for 20 min at 25°C to separate the hexadecane from the aqueous phase. Then, the lower aqueous phase was carefully aliquoted into a cuvette and the absorbance value (Acs) was measured. As control, 2 mL of *Candida* suspension was mixed with 2 mL of sterile saline in a sterile test-tube without the addition of nicotine. The cell surface hydrophobicity (CSH; percentage change in \( A_{550} \)) was calculated using the following formula [14]:

\[
\text{CSH} = \left( \frac{A_t - A_u}{A_u} \right) \times 100, 
\]

where \( A_t \) is the absorbance value at 550 nm of total cell suspension in the absence of hexadecane and \( A_u \) is the absorbance value at 550 nm of total cell suspension in the presence of hexadecane.

**Cell aggregation**

An exponential phase culture of each *Candida* sp. (1 x 10^6 cells/mL) was inoculated in YPD broth containing different concentrations of nicotine (1.0, 2.0, 4.0 and 8.0 mg/mL). Fresh YPD broth without nicotine was used as a negative control. The cells were harvested by centrifugation at 12,000 g and the cell pellet formed was washed twice with PBS buffer and suspended in the same buffer. From the *Candida* suspensions (1 x 10^6 cells/mL), 1 mL was vigorously mixed with 10 mL of PBS in sterile test-tubes and left at 25°C for 24 h. At every 6-h interval, 1 mL of the suspension was transferred into a fresh cuvette and the absorbance was measured at 595 nm. The speed of *Candida* cells sedimentation to the bottom of the tube and the percentage of aggregation with different concentrations of nicotine were calculated using the following formula [15]:

\[
\text{Aggregation percentage (\%)} = 1 - \left( \frac{A_t}{A_u} \right) \times 100, 
\]

where \( A_t \) is the absorbance at 550 nm of value at time \( t \) and \( A_u \) is the absorbance value at time \( t = 0 \).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

The *Candida* strains were respectively grown in YPD broth with 0, 1 and 2 mg/mL nicotine for 18 h. The cell pellet was harvested by centrifugation at 12,000 g and washed three times with saline. As stated in the instruction manual of the Easy-RED BYF Total RNA Extraction kit (Intron Biotechnology Inc., Seongnam, South Korea), approximately 5 x 10^6 cells/mL were extracted to be used in further experimental steps. The cells were suspended in 250 μL of prelysis buffer and incubated at 95°C. The RNA was isolated and purified by the easy-RED solution. The RNA concentration was determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Prime reverse transcription-polymerase chain reaction (RT-PCR) Premix 2X (GENET BIO), HWP1 (forward 5′-CCATGTGATGATTACCACA-3′ and reverse 5′-GCTGGACAAGAACGTCGAG-3′) and ALS3 primers (forward 5′-CCACAGCTTCCACTTCTCT-3′ and reverse 5′-TGAGATGACGATTACCACC-3′; 1 μL) [16], cDNA template (2 μL) and RNase-free water were processed for RT-PCR replication. Reserve transcription was performed at 42°C for 30 min to synthesize the cDNA followed by denaturation at 94°C for 10 min to deactivate the reserve transcriptase and activate the HS Prime Tag DNA polymerase. The samples were subjected to 30 cycles of denaturation (94°C), annealing (45–65°C) and extension (72°C), each for 30 s, followed by final extension at 72°C for 5 min (Eppendorf, Hamburg, Germany). Then, 6 μL of PCR product was used for separation by electrophoresis in 1% (w/v) of agarose gel and stained with ethidium bromide. The expression levels of the selected genes were visualized using ultraviolet (UV) illumination (Alphaimager 2200, Alpha Innotech, San Leandro, USA). ACT1 (forward 5′-GCGGTGGAGAGCATGACCACCAC-3′ and reverse 5′-GGAGTTGAAATGTTTGTG-3′) [16] was used as positive control to ensure
the Candida species are carrying the specific genes, respectively.

**Statistical analysis**

All data were computed and expressed as mean values with standard deviation (±SD) from three independent experiments performed in triplicate (n = 9). Independent t-test was carried out to compare two groups. Statistical analysis was performed using SPSS software (version 23.0). A p-value of less than 0.05 was considered to indicate statistically significant differences.

**Results and discussion**

**MIC/MFC and MBIC of nicotine against Candida species**

The MIC and MBIC assays were performed to determine the inhibitory effect caused by nicotine on planktonic and biofilm growth of C. albicans and C. parapsilosis. The results showed nicotine to have MIC 4 of mg/mL towards C. albicans and C. parapsilosis. The MFC values towards both species were 16 mg/mL. In our study, the MBIC of nicotine was 4 mg/mL against both tested Candida species. Thus, the data obtained revealed that nicotine exhibited a similar inhibitory effect on planktonic cells and biofilm of C. albicans and C. parapsilosis.

**Nicotine increases the planktonic cell growth of C. albicans and C. parapsilosis**

The growth of C. albicans and C. parapsilosis was observed to increase in the logarithmic phase when the nicotine concentration was 0–0.5 mg/mL. The growth of the Candida strains rapidly increased at nicotine concentrations of 1–2 mg/mL. The candidal count of C. albicans and C. parapsilosis showed an increase from 1 × 10^6 cells/mL to 7.0 × 10^8 cells/mL and to 5.0 × 10^8 cells/mL, respectively, at nicotine concentration of 2 mg/mL. The significant increase in viable cell counts indicated that the populations of oral microbes multiplied rapidly as the nicotine concentration increased. It is possible for the free-floating planktonic Candida cells to frequently attach onto the tooth surface upon exposure of nicotine. This may lead to Candida-related pathogenesis in the oral cavity. However, at 4 mg/mL, the growth of C. albicans and C. parapsilosis declined. The counts of C. albicans and C. parapsilosis were observed to decrease by about 50%–75% as compared to unexposed cells at 4 mg/mL of nicotine. At 8 mg/mL of nicotine, the growth of both Candida sp. was completely constrained, with approximately 90% reduction in the number of candidal cells compared to unexposed cells. This is in agreement with the general understanding that nicotine is a potentially toxic substance that could inhibit the cellular metabolism, resulting in changes of candidal growth in the formation of oral planktonic cells or biofilms.

Further, the specific growth rate and doubling time were calculated to compare the Candida sp. growth kinetics before and after nicotine exposure. The effect of nicotine on the growth profiles of Candida sp. was determined based on the continuous monitoring of optical density changes of fungal growth over time. From the data, the growth rates of C. albicans (0.503 ± 0.008 h⁻¹) and C. parapsilosis (0.777 ± 0.014 h⁻¹) were observed to be highest at 2 mg/mL nicotine when compared with other concentrations. C. parapsilosis showed the highest growth rates (0.680 ± 0.006 h⁻¹) indicating high proliferation compared to C. albicans. Interestingly, the doubling time of C. parapsilosis (7.872 ± 0.003 h) was observed to be slightly shorter than that of C. albicans (Table 1). In the present study, the growth of C. albicans and C. parapsilosis were observed to increase upon exposure to nicotine below 2 mg/mL, indicating it as a favourable environmental condition for proliferation of Candida species, as described by Arendorf and Walker [4]. However, nicotine concentrations higher than 2 mg/mL decreased the growth rate of Candida sp.

**Nicotine increases the formation of C. albicans and C. parapsilosis biofilm**

As a next step in our study, the potential of nicotine to influence the development of biofilm was studied (Figure 1). A biofilm is defined as a mass of microorganisms in which cells are frequently lodged within a self-produced matrix of extracellular polymeric substances (EPS) and adhere to each other or to a solid surface. The biomass of biofilm was measured using a microplate-based crystal violet assay. The readings were presented in reflective light units (RLU). Thus, the higher readings of RLU indicate higher biofilm formed. The biofilm mass

| Oral microbes          | Doubling time (g) and specific growth rate (µ) | Unexposed | 1 mg/mL | 2 mg/mL |
|------------------------|-----------------------------------------------|-----------|---------|---------|
| **Candida albicans**   |                                               |           |         |         |
| µ (h⁻¹)                | 0.453                                         | 0.455     | 0.503   |         |
| g (h)                  | 8.493                                         | 8.532     | 9.431   |         |
| Increase in µ          | –                                             | 0.002     | 0.050   |         |
| Increase in g          | –                                             | 0.039     | 0.938   |         |
| **Candida parapsilosis** |                                             |           |         |         |
| µ (h⁻¹)                | 0.680                                         | 0.736     | 0.777   |         |
| g (h)                  | 7.872                                         | 8.521     | 8.997   |         |
| Increase in µ          | –                                             | 0.056     | 0.097   |         |
| Increase in g          | –                                             | 0.649     | 1.125   |         |
of *C. albicans* without nicotine exposure was 0.105 ± 0.002 RLU, which was higher compared to that of *C. parapsilosis*, which demonstrated only 0.09 ± 0.003 RLU. Thus, this indicated that the *C. albicans* strain produces more biofilm than the tested *C. parapsilosis* strain. The formation of biofilm increased in ascending order as the concentration of nicotine increased to 0.5 mg/mL. However, we found that the biofilm mass was reduced at nicotine concentrations higher than 0.5 mg/mL. At 0.25 mg/mL nicotine, the formation of biofilm in *C. albicans* was 26.7% (0.133 ± 0.002 RLU), while 18.6% (0.115 ± 0.004 RLU) in *C. parapsilosis*. In total, the highest biofilm biomass, 37.1% (0.144 ± 0.001 RLU) and 20.6% (0.117 ± 0.006 RLU) of *C. albicans* and *C. parapsilosis*, respectively, was found to form at 0.5 mg/mL nicotine. A significant difference in biofilm formation at 0.25 and 0.5 mg/mL nicotine was observed in both *C. albicans* and *C. parapsilosis*. Nevertheless, exposure to 1 mg/mL nicotine led to 21% (0.127 ± 0.001 RLU) reduction in the biofilm mass of *C. albicans* followed by another reduction of 13.4% (0.113 ± 0.001 RLU) at 2 mg/mL. When *C. albicans* cells were treated with 4 mg/mL nicotine, the biofilm formation was reduced to 4% (0.082 ± 0.002 RLU) and further reduced to 3.8% (0.081 ± 0.001 RLU) at 8 mg/mL as compared to the unexposed control. Thus, the biofilm mass of *C. albicans* became constant at nicotine concentrations higher than 8 mg/mL. However, the biofilm mass of *C. parapsilosis* was reduced to 9.28% (0.106 ± 0.003 RLU) at 1 mg/mL and remained unchanged when treated with 2 mg/mL (0.106 ± 0.002 RLU) compared to the unexposed control. Further reduction in the biofilm mass was observed when the cells were treated with 4 mg/mL (0.086 ± 0.001 RLU) and 8 mg/mL (0.083 ± 0.002 RLU). The data showed that any further increase in nicotine concentration resulted in unchanged biofilm mass. Our data demonstrated that nicotine could more highly influence the growth of microorganisms in biofilm environment than in planktonic form. These results are in agreement with the suggestion that increased fungal growth as biofilm cells may be due to a broader surface area available for the attachment [17]. Many recent studies have indicated that biofilm is the preferred form of growth of most microbes in the oral cavity. Biofilms have significantly higher developed resistance against chemicals, antibodies, and various antimicrobials than planktonic cells [2,3,6,8].

**Adherence of *C. albicans* and *C. parapsilosis* induced by the presence of nicotine**

The results from the present study indicated that the two species, *C. albicans* and *C. parapsilosis*, can be categorized as highly hydrophobic (*p < 0.05*). The CSH values of both *Candida* sp. were found to increase (*p < 0.05*) upon exposure to 2 mg/mL of nicotine or less. Compared to unexposed cells, 1 mg/mL nicotine drastically increased the CSH of *C. albicans* by more than 82.32%, whereas 2 mg/mL could further increase the CSH up to 167.71% (Figure 2). However, at nicotine concentration higher than 2 mg/mL, the CSH of *C. albicans* was found to gradually decrease.

A similar pattern of reduction in CSH was also observed in *C. parapsilosis*. Following exposure to 1 mg/mL of nicotine, the *C. parapsilosis* cells exhibited the highest CSH value. However, at 2 mg/mL nicotine, a slight reduction in CSH was observed compared to the CSH value at 1 mg/mL nicotine. Further reduction was observed at the higher concentrations, 4 mg/mL and 8 mg/mL, of nicotine (Figure 2).

In our study, nicotine increased the CSH of *C. albicans* at 1 and 2 mg/mL. Thus, nicotine showed a relatively stronger effect on CSH of *C. albicans* than on that of

![Effect of nicotine on biofilm formation of oral candida](image)

**Figure 1.** Biofilm formation of *Candida albicans* and *Candida parapsilosis* after 24 h. The absorbance at 490 nm of crystal violet-stained biofilm at different nicotine concentrations is shown with mean and standard deviation after three independent experiments (*n* = 9).

![Effect of nicotine on the CSH of candidal cell](image)

**Figure 2.** Effect of nicotine on the CSH of *Candida* cells. The data are average values and standard deviation of three independent experiments (*n* = 9).
C. parapsilosis. The adhesion of yeast cells mediates the colonization and invasion of human tissues; thus, it is considered a crucial virulence factor in yeasts, especially in pathogenic Candida sp. [18]. The quality of hydrophobicity in cells can increase the proclivity of microorganisms for adherence. A high level of CSH indicated that more Candida cells would get attached to the tooth surface, which directly contributes to dental plaque. The data indicated that C. albicans cells were more hydrophobic than C. parapsilosis cells. This could possibly explain why C. albicans is more frequently isolated from the oral cavity than non-Candida albicans species particularly, C. parapsilosis [6,19]. This may imply that nicotine has the ability to alter the surface characteristics of the microorganisms as demonstrated and, thus, increase the adsorption to the hydrocarbon hexadecane. The stress induced by nicotine in the studied C. albicans and C. parapsilosis strains could increase the SCH, leading to enhancement in the cell adherence ability. Higher positive values of CSH indicate more hydrophobic amino acids located in a protein region, thus initiating a high level of oral biofilm formation. In addition, the presence of hydrophobic proteins in the polysaccharide matrix of the candidal cell wall contributes to the strength of adhesion receptors and increases the pathogenesis of yeasts [20]. Independent t-test showed that the amount of hexadecane absorbed into the C. albicans and C. parapsilosis cells exposed to nicotine was significantly different (p < 0.05) compared to that of unexposed cells.

Aggregation of Candida species increased upon exposure of nicotine

Besides cell extracellular polysaccharides and salivary components, direct cell-to-cell binding may also contribute to dental plaque. To determine the nature of surface components involved in cell-to-cell interaction leading to aggregation, the Candida strains were treated with nicotine. In our study, we observed that Candida cells tended to aggregate in an exposure-time-dependent manner.

It was observed that by 6 h of incubation in various concentrations of nicotine (1, 2, 4 and 8 mg/mL), about 10%–25% of C. albicans cells and 25%–40% of C. parapsilosis were aggregated. The cell aggregation increased to 25%–35% and 50%–80% in C. albicans and C. parapsilosis, respectively, at 12 h of incubation. Further increase in the aggregation of C. albicans and C. parapsilosis cells was observed at 24 h. Thus, the aggregation observed at 24 h of treatment was more pronounced than that at 12 h of treatment, at the corresponding nicotine concentrations in both C. albicans and C. parapsilosis (Figure 3).

High level of aggregation was found in C. albicans and C. parapsilosis with the addition of nicotine when compared to that of unexposed cells. At 1 mg/mL nicotine, 38.49% of C. albicans cells were aggregated compared to 77% in C. parapsilosis. Cell aggregation increased by 40.14% and 88% of C. albicans and C. parapsilosis at nicotine concentration of 2 mg/mL. Nicotine exposure led to further aggregation of C. albicans cells, up to 43.86%, at 4 mg/mL, which was the highest aggregation level. However, at 8 mg/mL of nicotine, the aggregation level was reduced to 31%. The cell aggregation in C. parapsilosis was reduced to 84% at 4 mg/mL nicotine followed by 79% at 8 mg/mL nicotine compared to that in unexposed cell cultures (Figure 3).

These results suggest that nicotine exposure could induce C. albicans and C. parapsilosis aggregation in a dose- and time-dependent manner. However, C. albicans was more sensitive to nicotine treatment compared to C. parapsilosis. This is in agreement with earlier reports that nicotine could escalate cell aggregation in oral microbes [8,17]. An increased aggregation indicated more Candida cells being able to accumulate and aggregate with each other by forming strong bonds between cell surface proteins. Thus, to further explore this effect, we carried out molecular analysis to evaluate the potential role of the HWP1 and ALS3 genes associated with the binding ability of Candida in the presence of nicotine.

Nicotine altered the expression of HWP1 and ALS3 of Candida cells

The regulation of HWP1 and ALS3 transcripts was analyzed in both C. albicans and C. parapsilosis, while ACT1 was used as the positive control. HWP1 and ALS3 have been reported to be involved in the adhesion of Candida
cells to tooth surfaces [10,11,16,21]. HWP1 has unique adherence characteristics, as it forms covalent bonds to proteins on human buccal epithelial cells in host tissues [10]. The results from our study showed that exposure of *C. albicans* and *C. parapsilosis* to nicotine affected the expression of gene transcripts with the increase of nicotine concentrations. Both *C. albicans* and *C. parapsilosis* expressed HWP1 transcripts in physiological growth conditions. In the presence of increasing concentrations of nicotine, the expression of HWP1 in both Candida strains was highly upregulated. It may be hypothesized that HWP1 probably forms more covalent bonds to the proteins on human epithelial cells, which enables more candidal cells to attach to host tissues. This may maximize the chances for the cells to propagate and sustain their existence in the oral cavity. In the presence of nicotine, the expression levels of HWP1 in *C. albicans* (Figure 4(a)) and *C. parapsilosis* (Figure 4(b)) increased in a concentration-dependent manner.

A similar trend was also observed for the ALS3 transcripts in *C. albicans* and *C. parapsilosis* (Figure 4(c)). Als3 is encoded by the ALS3 gene, which is a member of the agglutinin-like sequence (ALS) gene family. The main role of Als3 is to invade into the oral epithelial cells and endothelial cells by inducing epithelial cells endocytosis. It binds to host cell receptors such as E-cadherin and N-cadherin on oral epithelial cells to induce host cells to endocytose the microorganism [11]. Als3 is also required in biofilm formation and finally in the acquisition of iron from ferritin by hyphal cells [21,22]. In this study, both *C. albicans* and *C. parapsilosis* expressed ALS3, which is responsible for mediating attachment of candidal cells to epithelial and endothelial cells and to extracellular matrix proteins [21]. Our results also demonstrated that nicotine treatment of *C. albicans* and *C. parapsilosis* resulted in up-regulation of ALS3 expression. This explains the reason why more Candida cells could attach to oral epithelial cells as nicotine concentration increases [23]. The high expression levels of HWP1 and ALS3 in the studied Candida sp. indicate that nicotine could influence the level of adherence [24]. Both upregulated gene expression and adherence have become a virulence trait of candidal cells.

**Figure 4.** Expression of HWP1 in *C. albicans* (A) and *C. parapsilosis* (B) at nicotine concentrations of 1 and 2 mg/mL: Lanes 1 and 5, 100 bp DNA marker; Lanes 2 and 6, unexposed Candida sp.; Lanes 3 and 7, *Candida* sp. exposed to 1 mg/mL nicotine; Lanes 4 and 8, *Candida* sp. exposed to 2 mg/mL nicotine. Expression of ALS3 mRNA (C) in *C. albicans* (Lanes 2–4) and *C. parapsilosis* (Lanes 5–7) following nicotine exposure: Lanes 1 and 8: 100 bp DNA marker; Lanes 2 and 5: unexposed *Candida* sp.; Lanes 3 and 6: *Candida* sp. exposed to 1 mg/mL nicotine; Lanes 4 and 7: *Candida* sp. exposed to 2 mg/mL nicotine.

**Conclusions**

The results from the present study showed that nicotine stimulated *C. albicans* and *C. parapsilosis* planktonic cell growth, biofilm formation, aggregation and the expression of some genes encoding key binding-related proteins. In addition, the effects of nicotine on the studied microorganisms were dose-dependent. To the best of our knowledge, this is the first study to demonstrate the role of nicotine in causing up-regulation of HWP1 and ALS3 expression in *C. albicans ATCC 14053* and *C. parapsilosis ATCC 22019 in vitro*. It could be hypothesized that these effects might promote more *C. albicans* and *C. parapsilosis* cells to attach onto tooth surfaces and, consequently, to development of dental plaque in cigarette smokers.
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