Microorganisms play a role in oral mucositis after cancer therapy. The current study explored the hypothesis that Candida spp. alone and together with Porphyromonas gingivalis cause delayed healing of oral ulcerations due to the inhibition of wound closure. An in vitro scratch assay model was used to study the influence of viable and heat-killed Candida glabrata, Candida kefyr, and Candida albicans on cell migration of oral epithelial cells. Separately, the effect of conditioned medium of Candida spp. and the effect of a mixed infection of Candida spp. with P. gingivalis on wound closure was studied. In the presence of 10 viable C. glabrata or C. kefyr versus one epithelial cell, with a multiplicity of infection (MOI) of 10, the relative closure of the scratch was 26% and 17%, respectively. At a MOI of 1, this was 60% for C. glabrata and 78% for C. kefyr. The inhibition of oral epithelial cell migration challenged with either C. glabrata or C. kefyr together with P. gingivalis was stronger than the inhibition caused by one of both organisms separately. Candida spp. inhibit cell migration in vitro. A combination of Candida spp. and P. gingivalis inhibited cell migration more than either microorganism separately.

**KEYWORDS**
- Candida glabrata
- Candida kefyr
- Porphyromonas gingivalis
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signaling. They conclude that the altered oral microbiome has the potential to exacerbate the mucosal damage by potentiating apoptosis and the production of these pro-inflammatory cytokines [8].

In addition to bacteria, fungi may also be related to oral mucositis. In immunocompromised individuals, Candida albicans frequently overgrows the microbial flora and causes infections and epithelial damage [13,14]. Candida spp., particularly C. albicans, are associated with oral mucositis in patients with hematological malignancies [15,16]. In a study with head and neck cancer patients receiving radiotherapy, oral mucositis with pseudomembranous candidiasis was diagnosed in 77% (30/39) of the patients. C. albicans was the predominant species isolated, followed by Candida glabrata and Candida kefyr [14]. C. glabrata and C. kefyr were identified as positive predictors for mucosal ulcerations after hematopoietic stem-cell transplantation [10]. On the other hand, Westbrook et al. [17] and Epstein et al. [18] did not report a positive correlation between Candida colonization and the presence or severity of oral mucositis in stem-cell transplanted patients. The role of Candida spp. in the pathogenesis of oral mucositis remains to be elucidated in more detail [5].

The current study explored the effect of Candida spp. on epithelial cell migration in vitro. It was examined by using an in vitro scratch assay model, as previously described by Laheij et al. [12]. Epithelial cells were exposed to viable, heat-killed, and conditioned medium of Candida spp. From a previous study, it was already known that P. gingivalis is able to inhibit wound closure in vitro [12]. Therefore, the effect of a mixed infection of Candida spp. and P. gingivalis on epithelial cell migration was also studied. Finally, the effect of Candida spp. on oxygen saturation was studied, as it was hypothesized that the oxygen-reducing capacity of Candida spp. might be an important factor of interaction with P. gingivalis.

Materials and methods

Epithelial cells

The Japanese Collection of Research Bioresources (Osaka, Japan) provided the human buccal epithelial cell line H0-1-N-1. The cells were grown in an incubator at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air with Dulbecco’s modified Eagle’s medium Ham’s F-12 nutrient mixture (DMEM-F12, Invitrogen, Carlsbad, CA), completed with 10% fetal calf serum (HyClone, Logan, UT), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B (all from Sigma–Aldrich, St. Louis, MO). After confluency was reached over the entire 75 cm² bottle surface (Corning, New York, NY), the cells were detached using 0.25% trypsin-EDTA (Invitrogen) and counted with a hemocytometer. Finally, the cells were seeded in a 24-well plate in DMEM-F12 medium at a concentration of 3–5 × 10⁵ cells/mL.

Bacterial strains and culture

P. gingivalis ATCC 33,277 was cultured anaerobically (80% N₂, 10% H₂, and 10% CO₂) in Brain-Heart-Infusion (BHI; 37 g/L; BD Difco, Le Pont de Claix, France) supplemented with hemin (5 mg/L) and menadione (1 mg/L). C. glabrata CBS 138, C. kefyr CBS 1970, C. albicans BWP-17 (hyphal growth), C. albicans HGC-1 ΔΔ (knockout with pseudo-hyphae), and C. albicans HGC-1 (complementation strain) were cultured aerobically at 37°C in amino acid-depleted, glucose-enriched Yeast Nitrogen Base (YNB; 6.7 g/L; BD Difco). The rationale for the mutant and the complementary strain was to study the influence of hyphae formation on cell migration. All microorganisms were grown until log phase, which was ascertained by measuring the optical density (OD). Subsequently, the microorganisms were washed twice with Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen) and re-suspended in keratinocyte serum-free medium (SFM; Invitrogen) at a predetermined OD: P. gingivalis OD₆₀₀ of 0.1 (corresponding to 5 × 10⁵ colony forming units [CFU]/mL) [12], C. kefyr OD₆₀₀ of 0.7 (corresponding to 4 × 10⁶ CFU/mL), and OD₆₀₀ of 1.0 for C. glabrata and C. albicans (corresponding, respectively, to 4 × 10⁷ and 3 × 10⁷ CFU/mL). The corresponding CFUs were determined beforehand. For each scratch assay, a freshly prepared bacterial and/or yeast culture was used. All cultures were checked for purity and hyphal growth by culturing and Gram staining.

Heat-killed Candida

All Candida spp. were cultured and grown until log-phase and killed at 60°C for 60 min. The lack of viability was confirmed by plating cells on tryptic soy agar plates and incubating them at 37°C for 2 days. Gram staining was done to check for purity and hyphal growth and to confirm the presence of intact cell walls. After killing, the yeast cells were washed twice with DPBS and suspended in SFM at the required OD₆₀₀ (the same as viable yeast cells) and frozen at −80°C until use.

Conditioned medium

Again, all Candida spp. were cultured and grown until log-phase. After culturing, they were washed twice with DPBS, resuspended in SFM at the required OD₆₀₀ (the same as viable yeast cells), and incubated aerobically for 5 h. After centrifuging, the yeast cells were removed. The conditioned medium was filter
sterilized (0.2 μm; Sarstedt, Nümbrecht, Germany) and frozen at −80°C until use.

Wound closure assay

The cells were seeded at a density of 3–5 × 10^4 cells/mL in DMEM-F12 in 24-well plates and incubated until the cells reached confluency over the entire well surface. Before scratching a straight line in the monolayer of cells using a 1,000 μL blue pipette point (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), the cells were washed once with DPBS. To remove any detached cells, they were washed twice with DPBS after scratching. In the experiments with viable (monoinfection) heat-killed, and conditioned medium of Candida spp., the wells were treated with 1 mL of solution (prepared as described above) with a corresponding multiplicity of infection (MOI) of 10 and 1. The wells exposed to a mixed infection were treated with 0.5 mL of solution of both organisms that was twice as concentrated. One milliliter corresponded to a MOI of 10 and 1 for C. glabrata and C. kefyr and a MOI of 100 and 10 for P. gingivalis. The control group was treated with SFM. Immediately and after 17 h, the scratch was photographed using an inverted digital phase contrast microscope EVOS FL (Advanced Microscopy Group, Mill Creek, WA). The relative migration distance was calculated with the formula: 100 − [(scratch surface at 17 h/scratch surface at 0 h) × 100]. The surface of the scratch was calculated with Adobe Photoshop CS4 v11.0.1. The relative closure of the scratch under control conditions was 100%. Each treatment was performed in triplicate, and each experiment was performed on at least three separate occasions.

Oxygen consumption

C. glabrata and C. kefyr were cultured in YNB and resuspended in keratinocyte SFM. The oxygen consumption of both species was measured at an OD_600 of 0.5. Oxygen consumption was measured using two AppliSens dissolved oxygen sensors coupled to a Bio Controller ADI 1030 (Applikon Biotechnology, Delft, The Netherlands). The electrodes measured the percentage of dissolved oxygen in the fluid. All the measurements were conducted under aerobic conditions in an incubator set at 37°C. The bijoux tubes were not sealed airtight so that the exchange of oxygen between the fluid and the air was still possible.

Statistical analyses

Data of three or more separate experiments with similar conditions were assembled and analyzed. Friedman’s two-way analysis of variance was used to determine that the results of separate experiments had the same distribution of scores. Differences in relative closure of the scratch between different bacterial conditions were calculated with the nonparametric Mann–Whitney U-test. Statistical analysis was performed with IBM SPSS Statistics for Windows v23 (IBM Corp., Armonk, NY). A p-value of <0.05 was chosen as statistically significant.

Results

Influence of viable C. glabrata and C. kefyr on wound closure

The relative closure of the scratch challenged with C. glabrata and C. kefyr compared to control is shown in Figure 1. In the presence of 10 viable C. glabrata versus one epithelial cell (MOI of 10), the relative closure of the scratch was 26%. At a MOI of 1, the relative closure of the scratch was 60%. For C. kefyr at a MOI of 10 and a MOI of 1, the relative closure was 17% and 78%, respectively. All conditions led to significantly more inhibition of closure of the scratch compared to control. Figure 1 also shows the relative closure of the scratch caused by P. gingivalis, as studied by Laheij et al. [12]. There was no significant difference in the inhibition of cell migration between P. gingivalis at a MOI of 10 and C. kefyr at a MOI of 1 (p = 0.518) and P. gingivalis at a MOI of 100 and C. glabrata at a MOI of 10 (p = 0.077). An assay in which the epithelial cells were exposed to viable C. albicans spp. could not be conducted due to thick biofilm formation of C. albicans, which made the scratch invisible.

Influence of viable C. glabrata or C. kefyr with viable P. gingivalis on wound closure

The relative closure of the scratch exposed to a mixed infection of C. glabrata or C. kefyr and P. gingivalis is

![Figure 1. Relative closure (mean + standard error of the mean [SEM]) of scratched oral epithelial cells exposed to different concentrations of viable Candida glabrata, Candida kefyr, or Porphyromonas gingivalis compared to control. Difference with control is considered significant if p < 0.05 and is marked with an asterisk.](image-url)
shown in Figure 2a and b. WIt confirmed viability of both Candida spp. and P. gingivalis on several different occasions by culturing at the end of the experiments (data not shown) All combinations of C. glabrata or C. kefyr and P. gingivalis led to significantly more inhibition of closure of the scratch compared to control. Moreover, the migration of epithelial cells was inhibited stronger when challenged with a mixed infection than challenged with one of the microorganisms separately. This was independent of the MOI (Figure 3a and b). Generally, a dose–response effect of the microbial concentration was observed (). However, when a concentration of a MOI of 10 of either C. glabrata or C. kefyr was present, an increase in concentration of P. gingivalis from a MOI of 10 to a MOI of 100 did not lead to more inhibition of closure of the scratch (). So when both Candidas were present at a MOI of 10, the presence rather than the concentration of P. gingivalis was responsible for the additional inhibitory effect of the mixed infection on cell migration.

![Figure 2](image2.png)

Figure 2. Relative closure (mean + SEM) of scratched oral epithelial cells exposed to different concentrations of viable C. glabrata (A) or C. kefyr (B) with P. gingivalis compared to control. Difference with control is considered significant if \( p < 0.05 \) and is marked with an asterisk.

![Figure 3](image3.png)

Figure 3. The difference (mean + SEM) between a mono and a mixed infection of C. glabrata (A) or C. kefyr (B) with P. gingivalis. Difference is considered significant if \( p < 0.05 \) and is marked with an asterisk.
Influence of conditioned medium of and heat-killed C. glabrata, C. kefyr, and C. albicans on wound closure

The relative closure of the scratch in epithelial cells challenged with heat-killed Candida spp. did not differ from control. From conditioned medium only C. albicans BWP-17, a MOI of 10 did inhibit the relative closure of the scratch slightly, though significantly, compared to the control group (x = 88.9 vs. x = 100; p = 0.015).

Oxygen consumption of C. glabrata and C. kefyr

The graphs in Figure 4a and b display the average normalized oxygen concentration in SFM. Both C. glabrata and C. kefyr caused a clear decrease in oxygen over a relatively short period of time. The relative decrease in oxygen by C. kefyr was greater and more rapid compared to C. glabrata.

Conclusion

In our previous clinical study, P. gingivalis in particular, and at the same time C. glabrata and C. kefyr, were identified as explanatory variables of oral ulcerative mucositis in patients undergoing hematopoietic stem-cell transplantation [10]. In an in vitro scratch assay, a model for wound healing, it was found that P. gingivalis inhibited epithelial cell migration, thereby possibly delaying the healing of oral ulcerations in vivo [12]. The current study found that Candida spp. were able to inhibit cell migration too when using the same in vitro scratch assay. To the authors’ knowledge, this is the first study to report an inhibitory effect of C. glabrata and C. kefyr on the migration of oral epithelial cells in vitro. Unfortunately, it was not possible to study the effect of viable C. albicans on cell migration due to thick biofilm formation.

Interestingly, in this study, heat-killed C. glabrata, C. kefyr, and C. albicans and conditioned medium from these species did not inhibit cell migration. From this, it can be concluded that the inhibitory effect of these Candida spp. is dependent on a certain minimal cell-bound enzymatic or metabolic activity and is associated with temperature sensitive cell-wall proteins. The inhibitory effect is not associated with secreted metabolites and signaling molecules, or with temperature unsensitive cell-wall proteins. This is in contrast to conditioned medium of P. gingivalis and heat-killed P. gingivalis, which did inhibit epithelial cell migration [12].

The effect of a mixed infection of Candida spp. and P. gingivalis on the inhibition of epithelial cell migration was also studied. The inhibition of cell migration challenged with a mixed infection was stronger than the inhibition caused by one of both microorganisms separately. The inhibiting effect might partly be attributable to the oxygen-reducing effect of both Candida spp. Within a biofilm, bacterium–fungus interactions influence the overall survival and proliferation of the respective species [19]. C. albicans promotes growth and biofilm formation of anaerobic bacteria under aerobic conditions [20,21]. An explanation for this might be that Candida creates a pro P. gingivalis anaerobic microenvironment by using oxygen for its own metabolic processes (metabolic interaction). A fast and large reduction of oxygen was observed in SFM medium if C. glabrata or C. kefyr was present. Moreover, the lower oxygen levels might influence the viability of epithelial cells. Hieke et al. found that under anoxic conditions, the cell count of gingival epithelial cells was reduced to 75% after 24 h, 60% after 48 h, and 30% after 72 h compared to aerobically cultivated cells. Moreover, they noticed that the metabolic activity of the epithelial cells was reduced. However, the influence of oxygen levels on cell viability was not subject of the present study [22].

Epithelial cell death was not excluded as a mechanism of inhibition of epithelial cell migration in this study. However, during all experiments, the epithelial cells were strongly attached to the surface, and the cells looked morphologically viable. Moreover, in a previous study, using the same model, epithelial cell viability was confirmed [12]. Therefore, epithelial cell death would not appear to be the mechanism that is responsible for the inhibition of cell migration by Candida spp. that was observed in this study.

The present study found that the presence rather than the concentration of P. gingivalis was important for the additional inhibitory effect on cell migration when both Candida spp. were present at a MOI of 10. First, it is
possible that the inhibitory effect on epithelial cell migration of Candida and P. gingivalis is at its maximum at a MOI of 10 within the model that was used. Another explanation might be that one Candida cell can only interact with a certain amount of P. gingivalis, which means that after a certain threshold, extra P. gingivalis does not result in an additional effect. In the current study, C. glabrata and C. kefyr were found to be inhibitors of wound closure. This study supported the previously reported clinical data, in which Candida spp. were identified as positive predictors of oral ulcerative mucositis in hematopoietic stem-cell transplant recipients [10]. Both findings support the idea that Candida spp. might play a role in the complex process of ulcerative oral mucositis.

In conclusion, C. glabrata and C. kefyr strongly inhibited the migration of oral epithelial cells in an in vitro scratch assay. Moreover, a combination of Candida spp. and P. gingivalis inhibited cell migration more than either microorganism separately. In this interaction, Candida might play an essential role by creating a pro P. gingivalis anaerobic microenvironment. Heat-killed C. glabrata, C. kefyr, and C. albicans and conditioned medium from these species did not inhibit cell migration.

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