Acta Microbiologica et Immunologica Hungarica 63 (4), pp. 387–403 (2016)
DOI: 10.1556/030.63.2016.023
First published online December 8, 2016

EFFECTS OF HEMIN, CO_2, AND pH ON THE BRANCHING OF *CANDIDA ALBICANS* FILAMENTOUS FORMS

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(Received: 4 July 2016; accepted: 21 October 2016)

Morphological transitions of wild-type and oxidative stress-tolerant *Candida albicans* strains were followed in the RPMI-FBS culture medium at pH values and CO_2 levels characteristic for the anatomical niches inhabited by this opportunistic human pathogen fungus, including the oral cavity as well as the intestinal and vaginal lumens. Selected cultures were also supplemented with hemin modeling bleedings. Germination as well as elongation and branching of hyphae were monitored in the cultures using time-lapse video microscopy. Unexpectedly, branching time, which is defined as the time taken until the first branch of hypha emerges for the first time after germination, correlated well with alterations in the environmental conditions meanwhile no such correlations were found for germination time (time lasted until the

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appearance of the germination tube). Based on these observations, hypotheses were set up to estimate the significance of branching time in the pathogenesis of both superficial and systemic candidiases.

Keywords: *Candida albicans*, hemin, hyphal branching, morphological transitions, time-lapse video microscopy

Introduction

The dimorphic fungus *Candida albicans* is a commensal microorganism, which has been identified in healthy mycobiomes of the human skin, mouth, and gut [1]. In addition, *Candida* species with the predominance of *C. albicans* also appeared in 64.5% of reproductive-age asymptomatic women as part of their vaginal mycobiome [2]. In addition, *C. albicans* is the most common opportunistic fungal pathogen of humans, and it is also the causative agent of oral [3], gastrointestinal [4], and vulvovaginal [5] candidiases.

C. albicans is a dimorphic fungus, which is able to form hyphae (H) and pseudohyphae (PH) and, as a result, to penetrate epithelial cell layers and deep tissues. Its yeast (Y) form may disseminate in the bloodstream and also in other body cavities [6]. Y → H/PH morphological transitions are important virulence factors, which play very important roles in colonization of the human body by *C. albicans* [6–8].

The morphological transitions of *C. albicans* are influenced by various environmental factors. Y → H/PH switches are induced by temperatures between 35 °C and 42 °C [8, 9], blood serum [10], hemoglobin at 1 mg/ml concentration (approximately 15.5 μM with 62 μM heme content; [11]), hemin (protoporphyrin IX with ferric ion or oxidized heme, which is released from hemoglobin; [12]) at 5–100 μg/ml (equals to 7.7–153.4 μM; [13]) or at 50 μM (equals to 32.6 μg/ml) concentrations [14], CO_2 [15, 16], hypoxia + high CO_2 level [17], alkaline pH [15, 18], the available carbon sources [8], and nitrogen starvation [18].

C. albicans is able to colonize mucosa as a pathogenic fungus in a wide range of pH and CO_2 values [16, 19]. For example, the mucosal pH in the oral cavity varies between 6.28 ± 0.36 (buccal mucosa) and 7.34 ± 0.38 (palate) with a mean value of 6.78 [20]. Meanwhile, the gastrointestinal tract shows wide differences in pH considering values reported for the stomach (extremely acidic like pH 2.0) and the intestine (pH ~6.0–8.0) [16, 21, 22]. It is noteworthy that a slightly alkaline pH range of 7.5–8.0 was reported for secretions of the small and large intestinal epithelial cells [22]. *Candida* cells disseminating from the gastrointestinal tract (with pH values between 2.0 and 8.0) and entering the bloodstream

(pH 7.4) may cause systemic mycoses [23, 24]. Considering the CO_2 levels in the intestine, they may vary within the range of 5.1%–29% (v/v) in a basically hypoxic (0.1%–2.3% O_2), N_2-dominated (23%–80%) atmosphere [25]. The average pH of the vaginal lumen fluctuates between 4.2 (on day 14) and 6.6 (on day 2) with CO_2 values of 6.1% (46 mmHg on day 2) to 8.3% (62 mmHg on day 4) during the menstrual cycle [26].

Besides environmental factors, the acquisition of iron plays a central role in fungal metabolism and, therefore, it is considered as a key element in the invasion of host organisms by pathogenic fungi including *C. albicans* [14, 27]. In the human body, iron is present exclusively in bound forms, e.g., in hemoglobin, transferrin, and ferritin [14, 27–30]. Nevertheless, *C. albicans* has a remarkable arsenal to extract iron from its hosts, including the internalization and degradation of hemoglobin and hemin [14, 28, 31–33]. In the circulating blood, free hemoglobin and hemin cannot be found under normal physiological conditions due to the hemoglobin-binding and heme-binding capacities of haptoglobin and hemopexin, respectively [18]. On the other hand, hemoglobin is released frequently from the mucosa of the oral cavity, gastrointestinal tract, and vagina as a consequence of various infections and medical conditions and procedures [11, 29, 34].

In this study, we aimed at answering the intriguing question how varying physiological conditions sensed by *C. albicans* in the human body, e.g., acidic and alkaline pH values, changes in CO_2 levels, and the presence of hemin as well as the combination of these conditions, influence the morphological transitions of wild-type and oxidative stress-tolerant mutant strains of the dimorphic fungus. Using a time-lapse video microscopy technique, variations in the germination times and the times spent until the emergence of the first hyphal branch (branching times) under different environmental conditions have been compared. Unexpectedly, while variations in the germination times were largely strain dependent, the significant differences recorded in branching times clearly correlated with certain combinations of the studied environmental conditions. Hypotheses have been set up to evaluate the significance of these observations in the pathogeneses of various types of *Candida* infections.

Materials and Methods

C. albicans *strains and growth conditions*

C. albicans strains used in this study are as follows: SC5314, ATCC 14053, 4774, AF06, and 4774T. Strain 4774 is a clinical isolate from urine [35], whereas strains AF06 and 4774T are oxidative stress-tolerant mutants developed from

strains ATCC 14053 and 4774, respectively. The mutants were characterized with reduced hypha formation and delayed growth [35, 36]. All strain collections are available in the Department of Biotechnology and Microbiology, University of Debrecen, Debrecen, Hungary.

All strains, except SC5314, were precultured in 5-ml Sabouraud dextrose broth [2% (w/v) glucose and 1% (w/v) mycological peptone; pH 5.6] at 37 °C for 18 h as described by Fekete et al. [35, 36]. For preculturing the SC5314 strain, yeast extract peptone dextrose medium [2% (w/v) glucose, 1% (w/v) yeast extract, and 2% (w/v) mycological peptone] was used following the protocol of Jakab et al. [37]. Cells from these overnight cultures were collected by centrifugation (5 min, 4,000 rpm, 4 °C), were washed three times with phosphate-buffered saline [35–37], and were used to inoculate a 5-ml fresh bicarbonate-free RPMI 1640 medium (PAA The Cell Culture Company, Germany; [38]) containing 10% (v/v) fetal bovine serum (FBS; EUROCLONE, Italy; containing 13.42 mg/l hemoglobin, which equals to approximately 0.208 μM concentration with 0.832 μM heme content) (RPMI-FBS medium; [39, 40]) in polystyrene Petri dishes. The RPMI-FBS medium supports the filamentous growth of *C. albicans* [40]. The starting cell density was always 0.5×10^6 cells/ml, and the pH of the medium was set to 4.2, 5.3, 7.0, or 8.0 depending on the experiments. In a separate set of experiments, the RPMI-FBS medium was also supplemented with 50 μg/ml hemin (Sigma; 76.7 μM concentration; [13]). Cultures were incubated at 37 °C for 5 h either with 5% (v/v) or with 10% (v/v) CO_2 or without any CO_2 supplementation depending on the experiment.

Time-lapse video microscopy

Image sequences were recorded with a Carl Zeiss GFL Standard Microscope (Jena, Germany), which was equipped with a 10×0.25 objective and was modified for inverted usage with transmitted white light illumination. The equipment was set up in a Contherm MITRE 4000 CO_2 cell culture incubator [40, 41], and image acquisition occurred every 40th second for over 4 h with 28 ms exposure time, using a UCMOS01300KPA ocular camera driven by ToupView 3.2 software at SXGA (1,280 × 1,024) resolution (ToupTek Photonics, Hangzhou, China). Cells were only illuminated during image acquisition periods. Image stacks were saved into individual folders automatically with time tags in the filenames. Hypha and pseudohypha formations were characterized by germination time (the time elapsed to germination) and branching time (the time elapsed from germination to the first branching). In certain cases, when hyphal growth was parallel to the button of the Petri dishes, the lengths of hyphae measured from

germinating yeast cells to the first branches were also determined using the National Health Institute ImageJ software bundle (http://rsbweb.nih.gov/ij/docs/install/windows.html). Mean ± SD values calculated from data originated from 8 to 23 cells are presented for each set of experiments.

Statistical analyses

Pairwise comparisons were performed using two-tailed Welch's *t*-tests [42]. The resulting *p* values were adjusted for multiple comparisons with the Holm's procedure [43]. Statistical analysis was performed using the 3.1.2 version of the R statistical environment [44].

Results

A number of cell morphology-related parameters including the type of filamentous growth (pseudohypha or hypha), germination time, branching time, length of hypha from the hyphal apex to the first hyphal branch were monitored in *C. albicans* cultures under various physiological conditions that are characteristic for the human anatomic niches frequently occupied by *C. albicans*, e.g., the oral cavity and the intestinal and vaginal lumens. These morphological parameters are listed in Supplementary Material 1.

According to the experimental data collected by time-lapse video microscopy, yeast cell size remained constant prior to germination (data not shown), and once the cells adhered to the culture vessel's (Petri dish) bottom surface, germination began immediately. During germination, *C. albicans* cells kept some degrees of freedom to move and a fraction of germinating cells showed temporary deadherence from the surface. Hyphae showed twisted growth and the first branching typically emerged close to the basal end of germ tubes or even from the yeast cell itself as a second germ tube. The branching patterns of *C. albicans* were the same as published previously by Barelle et al. [45] and, hence, they are not presented here in more detail. All studied *C. albicans* strains formed hyphae in the RPMI-FBS medium at pH 7.0 and 8.0 independently in the presence of hemin or the applied CO_2 tension (Figure 1, Supplementary Material 1) without any detectable budding. In contrast, acidic pH values set at 4.2 or 5.3 completely inhibited hypha formation and supported pseudohyphal growth in all cases without the formation of yeast morphology cells (Figure 1, Supplementary Material 1).

Variations in the pH or CO_2 levels and the inclusion of hemin in the culture medium had significant effects on the germination times in many cases, but the

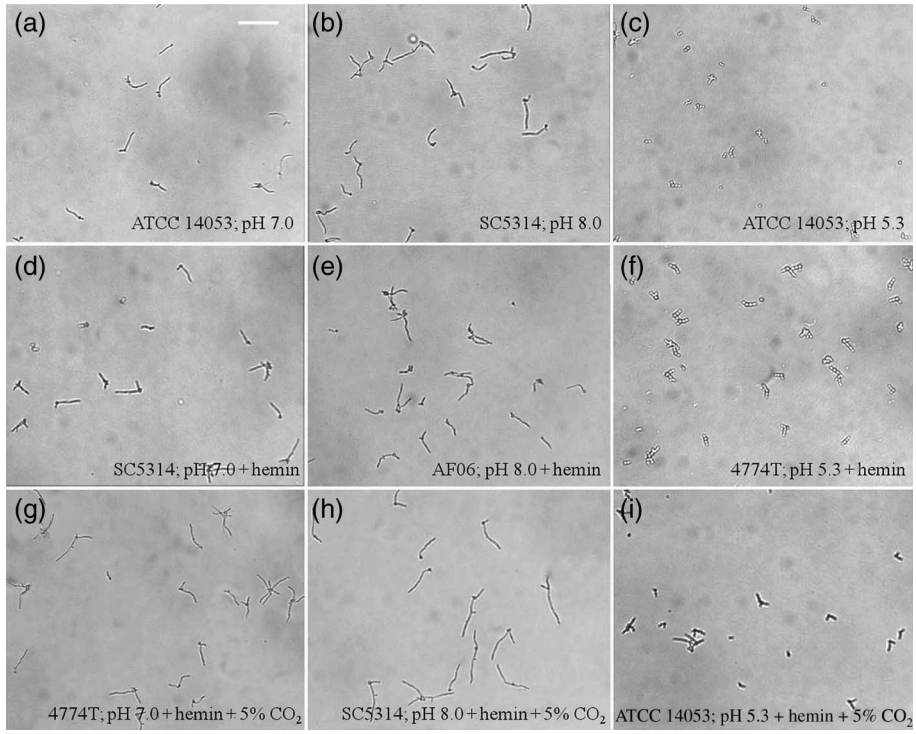

Figure 1. Typical morphological forms of *C. albicans*. Morphological forms of *C. albicans* grown in acidic (pH 5.3; parts c, f, and i), neutral (pH 7.0; parts a, d, and g), or alkaline (pH 8.0; parts b, e, and h) RPMI-FBS media supplemented with 50 μg/ml hemin (parts e–i), with 5% CO_2 (parts g–i) or grown without any hemin (parts a–c) or CO_2 (parts a–f) supplementations. Representative time-lapse video microscopic images were selected for the following *C. albicans* strains after 4 h incubations at 37 °C: SC5314 (parts b, d, and h), ATCC 14053 (parts a, c, and i), AF06 (part e), and 4774T (parts f and g). Bar = 100 μm

changes were rather strain specific and, hence, no clear parameter-dependent tendencies were observed (Supplementary Materials 1 and 2).

In contrast to the germination time, the changes in the branching time showed clear tendencies depending on the culture conditions employed (Figure 2, Supplementary Materials 1 and 2). For example, the supplementation of 5% (v/v) CO_2 increased the branching time at pH 7.0 in the presence of hemin in most cases (Figure 2, Supplementary Materials 1 and 2). In addition, changing the pH from 7.0 to 8.0 significantly increased the branching time in the case of 4 out of 5 strains [from 1.37 (ATCC 14053) to 1.90 (AF06) fold increases; no significant (1.03 fold) increase with strain 4774] grown with hemin but without CO_2 supplementation (Figure 2, Supplementary Materials 1 and 2). Comparing multiple conditions

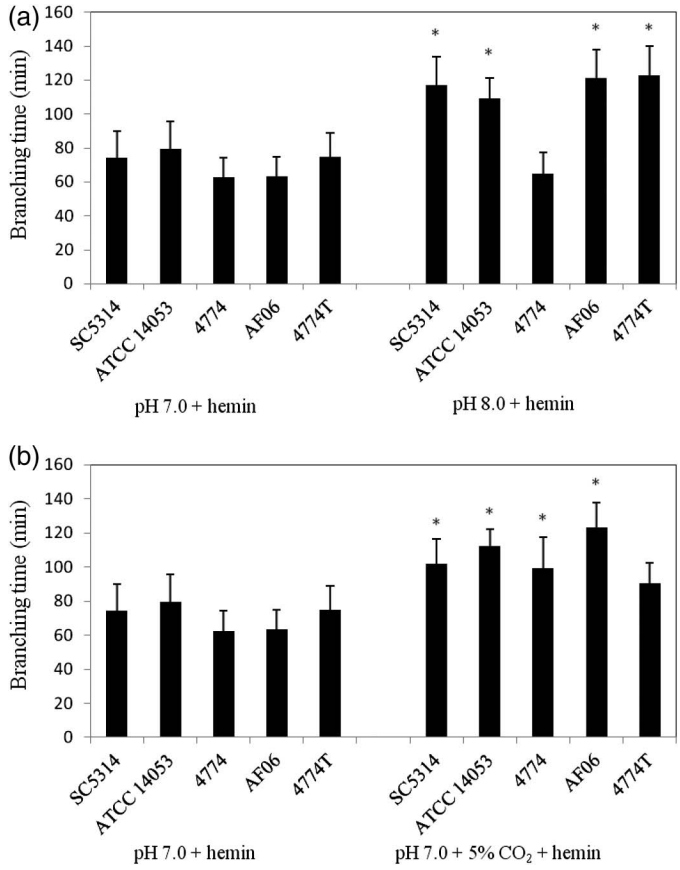

Figure 2. Effect of pH values (pH 7.0 vs. 8.0; part a) and carbon dioxide (no CO_2 vs. 5% CO_2 supplementation; part b) on the branching times of *C. albicans* cells. Significant differences at $p < 0.05$, calculated by the Holm's test, are indicated by asterisks

applied in our experiments, the shortest branching times were detected at pH 7.0 in the presence of hemin in cultures without CO_2 supplementation, and increase in the pH and/or 5% (v/v) CO_2 supplementation increased the branching times. However, this increase was not significant in all cases (Figure 2, Supplementary Materials 1 and 2). Culturing oxidative stress-tolerant *C. albicans* mutants (strains AF06 and 4774T) at 37 °C in the RPMI-FBS medium promoted filamentous growth and induced either pseudohypha (at pH 4.2 and 5.3) or hypha (at pH 7.0 and 8.0) formations similar to the parental strains (strains ATCC 14053 and 4774, respectively). These observations indicated clearly that phenotypic changes in

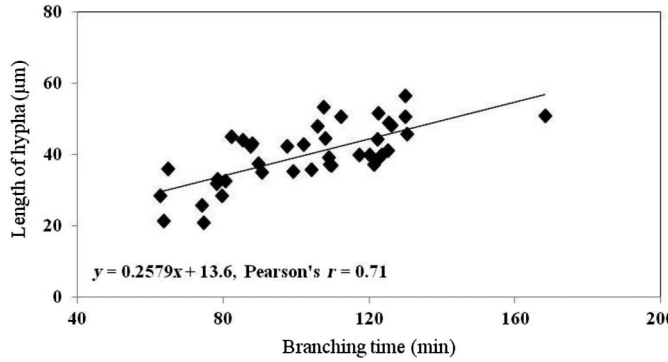

Figure 3. Comparison of hyphal growth-related morphological parameters measured in *C. albicans* cultures grown at neutral and alkaline pH values. The correlation between the average branching times and the average lengths of hyphae determined in each culture was characterized by Pearson correlation coefficient (Pearson's *r*)

stress tolerances did not influence the germination and branching times of *C. albicans*.

Importantly, branching times showed strong correlation (Pearson correlation coefficient = 0.71) with the lengths of hyphae at the time when the first branch was formed under all culture conditions analyzed (Figure 3, Supplementary Material 1). Unlike branching times, the growth rates of *C. albicans* hyphae were not influenced by the pH (7.0 or 8.0), the CO_2 levels (0% or 5%), or the presence of hemin because longer branching times were associated with longer hyphal segments, and thus, with similar hyphal growth rates.

Discussion

C. albicans, a wide-spread commensal pathogen of humans, has the capability to colonize or even invade a number of anatomical niches within the human body [1, 46]. It is well documented that the proper timing and the rate of Y → H and H → Y morphological transitions are the prerequisites of a successful invasion of the host by *C. albicans* [35, 36, 39, 47]. Any disturbance in the formation of the hypha may result in the failure of invasive growth and reduced virulence [48, 49]. Very importantly, yeast cells and true hyphae are always observed during *C. albicans* infections, such as oral candidiasis [50] or invasive candidiasis in the spleen, kidney, and liver [51, 52]. While true hyphae play an important role in invasiveness [6, 53], pseudohyphae are less virulent in hematogenously disseminated candidiasis [54] and their functions in infections are still

understudied [53]. Nevertheless, pseudohyphae may also contribute to the penetration of host tissues by the invading fungus [6, 55, 56].

In our experiments, culturing *C. albicans* at 37 °C in the RPMI-FBS filamentation medium [40] supported hypha (at pH 7.0 and 8.0) or pseudohypha (at pH 4.2 and 5.3) formations independently in the presence of hemin or CO_2 supplementation (Figure 1, Supplementary Material 1).

Among the parameters used to characterize hypha and pseudohypha formations, branching time of hyphae was dependent on culture conditions, e.g., pH values, CO_2 supplementation, and addition of hemin (Figure 2, Supplementary Materials 1 and 2). Meanwhile, changes in the germination times elicited by changes in the environmental conditions were strain specific rather than dependent on the varying conditions (Supplementary Materials 1 and 2). Furthermore, branching time was a more easily accessible parameter in terms of time-lapse video microscopy techniques and, importantly, the monitored hyphae were not required to grow parallel with the bottom surface of the culture vessel (in contrast to hyphal length measurements).

Taking into consideration, the clear-cut correlation between branching times and hyphal lengths measured at branching times (Figure 3, Supplementary Material 1), i.e., changes in the culture parameters did not influence significantly the growth rates of hyphae either, we concluded that the easily accessible branching time was a more suitable parameter to describe the physiological status of the *C. albicans* cells than either the germination time or the hyphal growth rate.

Considering the impact of fungal physiology on the formation of hyphal branches, it has been shown that the availability of nutrients regulates the branching of hyphae, namely, nutrient limitation decreases branching frequency and also promotes fungal invasion of host tissues [45, 57]. Therefore, increased branching is likely to indicate optimal physiological conditions for the fungus concomitant with relatively decreased invasiveness.

The combined effects of pH, CO_2, and hemin on hyphal branching are summarized in Figures 2 and 4. Interestingly, culturing *C. albicans* in the presence of hemin at pH 7.0 without CO_2 supplementation was characterized by the shortest branching times, and increased branching times were observed at pH 8.0 in the presence of hemin and at pH 7.0 with 5% CO_2 supplementation (Supplementary Materials 1 and 2). It is noteworthy that only Y → PH morphological transitions were observed at acidic pH values of 4.2 and 5.3 and in the presence of 5%–10% CO_2.

These observations on hyphal branching inspired us to set up the following hypotheses, which may provide us with suitable models to answer the intriguing question that how *C. albicans*, which inhabits so many anatomical niches within the human body, can cause various types of candidiasis [1, 46].

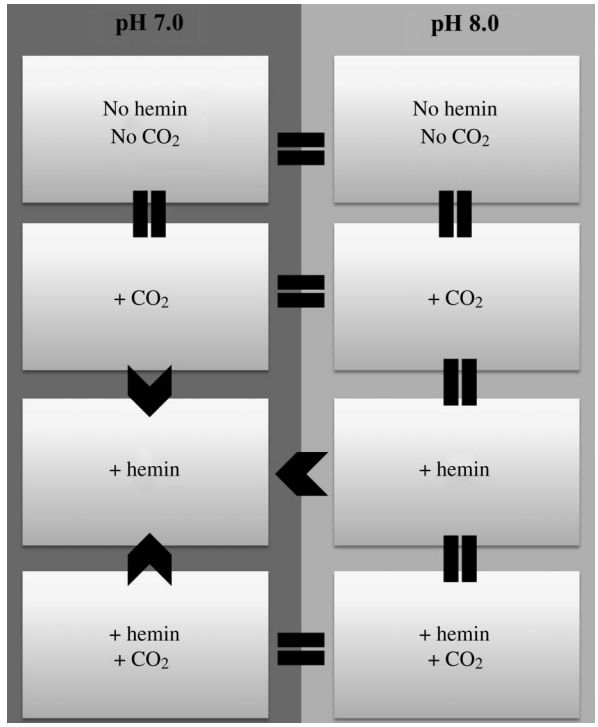

Figure 4. Shifts in the times elapsed until the first branching measured under various culture conditions (pH 7.0 and 8.0; no CO_2 vs. 5% CO_2 supplementation; and the addition of 50 μg/ml hemin)

(i) We assume that the combination of neutral pH, hemin, and no CO_2, which gives the shortest branching time (Figures 2 and 4), is a suitable model of the oral cavity [20], where bleeding occurs frequently as a result of a wide spectrum of pathological conditions [3, 58, 59]. It is important to note that hemin is released by oxidized ferric (Fe^{3+}) hemoglobin in the human body [60], and hemin has been demonstrated to initiate the Y → H morphological transition of *C. albicans in vitro* [13, 14], which is likely to facilitate the superficial invasion of the oral mucosa by *C. albicans in vivo* [50, 61, 62]. On the other hand, highly branched mycelium can be the prerequisite of an effective nutrient assimilation and may also moderate the invasiveness of *C. albicans* [45] in the oral cavity as well.

(ii) Upon reaching deeper tissues and blood vessels by *C. albicans* hyphae, the pH and the CO_2 concentration are expected to raise, which should result in a higher branching time and, hence, less-branched mycelium (Figures 2

and 4, Supplementary Materials 1 and 2). In fact, *C. albicans* hyphae visualized in infected tissues are very sparsely branched [6, 36, 51, 52, 63] because the subapical compartments of hyphae are arrested in the G1 phase of the cell cycle [12, 45].

(iii) Increasing pH (to 8.0) or CO_2 concentration (to 5% at pH 7.0) in the presence of hemin also increases the branching time (Figures 2 and 4, Supplementary Materials 1 and 2), which indicates that under environmental conditions reminiscent of that found in the intestine especially in the case of bleeding/ulceration [16, 22, 64–66], the invasiveness of *C. albicans* should increase. Further studies are needed to answer the intriguing question if the nutrients available in the intestinal lumen and the composition of the intestinal gases (e.g., the considerably decreased O_2 tension) would support predominantly yeast morphology with a commensal lifestyle or also Y → H morphological transitions preparing the fungus for the invasion of intestinal mucosa [24, 67].

(iv) The RPMI-FBS medium with acidic pH values of 4.2 and 5.3 and the presence of 5%–10% CO_2 can be a simplified model of the conditions *C. albicans* senses in the vaginal lumen, where both the pH (4.2–6.6) and the CO_2 tension (6.1%–8.3%) change periodically during the menstrual cycle [26]. Based on our data, we hypothesize that acidic pH (4.2 or 5.3) inhibits Y → H morphological transitions, initiates Y → PH switches instead and, hence, may protect the vaginal mucosa against invasive *C. albicans* infection during days 4–14 of the menstruation cycle (Figure 1, Supplementary Material 1). On the other hand, the elevation of pH under menstruation (up to pH 6.6), the relatively high CO_2 levels (6.1% on day 2 of the menstruation cycle) together with the hemoglobin content of the menstruation (uterine) fluid [68] may facilitate hypha formation and even invasion by *C. albicans*. One possible mechanism defending vaginal mucosa against microbial infections during menstruation can be the *Candida* secreted aspartic peptidase-dependent liberation of antibacterial peptide fragments called hemocidins from hemoglobin [69]. Considering that hemocidins showed only very weak activity toward *C. albicans*, further research is needed to shed light on the mechanisms controlling *Candida* spp. during menstruation.

From a medical point of view, various drug treatments, diabetes, surgery, polytrauma, catheters, neutropenia, have been reported to change pH values and CO_2 concentrations in the body fluids, which may aggravate further these major risk factors for invasive candidiasis [15, 23, 26, 53]. Therefore, future anti-*C. albicans* therapies may also include the reconstruction of physiologically relevant pH values and CO_2 concentrations at the sites of infections to prevent

or at least hinder the spread of *Candida* cells toward deeper tissues. Another therapeutic approach may rely on the disturbance of the hemoglobin and hemin uptake by *C. albicans* through hemoglobin/heme-binding receptors on the surface of *Candida* cells [32, 70, 71], which may also reduce the virulence of *C. albicans* [33]. It is worth noting that the dimorphic morphological transitions of *C. albicans* is an extensive studied area in fungal biology and are considered as promising targets in future antifungal drug research [72]. Time-lapse video microscopy may provide us with further important details of these transitions including the emergence of hyphal branches.

In conclusion, the application of time-lapse video microscopy helped us to introduce a new parameter, namely branching time, to characterize/estimate the morphological, physiological, and virulence status of *C. albicans* hyphae under various environmental conditions, which can be observed in different anatomical niches in the human body. In addition, the comparative analysis of the branching time values led us to build up simple models and hypotheses to try to explain the differences observable in the physiology and pathogenicity of *C. albicans* cells inhabiting the oral cavity as well as the intestinal and vaginal lumens. We hope that such hypotheses will help researchers working in this field to carry out further experiments both *in vitro* and *in vivo* to gain a deeper insight in the armory of this fungus to colonize and invade the human body, which will eventually lead to the development of novel anti-*C. albicans* agents and therapies.

Acknowledgements

This work was supported by the Hungarian Scientific Research Fund (grant numbers OTKA K75883 and K112181) and the National Development Agency (grant numbers TÁMOP-4.2.2.A-11/1/KONV-2012-0045 and TÁMOP-4.2.6-15/1-2015-0001). The TÁMOP projects were implemented through the New Hungary Development Plan, co-financed by the European Social Fund and the European Regional Development Fund.

Conflict of Interest

The authors declare no conflict of interest.

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