

# How does temperature trigger biofilm adhesion and growth in *Candida albicans* and two non-*Candida albicans* *Candida* species?

Debora Casagrande Pierantoni<sup>1</sup> | Laura Corte<sup>1</sup> | Arturo Casadevall<sup>2</sup> | Vincent Robert<sup>4</sup> | Gianluigi Cardinali<sup>1</sup>  | Carlo Tascini<sup>3</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

<sup>2</sup>Johns Hopkins Bloomberg School of Public Health, JHSPH Molecular, Microbiology & Immunology; JHUSOM,, Baltimore, MD, USA

<sup>3</sup>University Hospital "S. Maria della Misericordia" - Clinic of Infectious Diseases, Udine, Italy

<sup>4</sup>Westerdijk Fungal Biodiversity Institute, AD Utrecht, The Netherlands

## Correspondence

Gianluigi Cardinali, Department of Pharmaceutical Sciences, University of Perugia, Borgo 20 Giugno n. 74, 06121, Perugia, Italy.  
Email: gianluigi.cardianli@unipg.it

## Abstract

**Background:** Biofilm formation on biotic and abiotic surfaces is finely regulated by genetic factors but also by oxygen concentration, pH, temperature and other environmental factors, already extensively explored for bacterial biofilms. Much less is known about fungal biofilm, that is considered a virulence factor for *Candida* pathogenic species among the few fungal species able to grow and survive at high temperatures such as 37°C as well as those induced by fever. The resistance to high temperatures coupled with the ability to form biofilm are threatening factors of these fungal species that could severely impact at an epidemiological level.

**Objectives:** In this framework, we decided to study the thermal tolerance of biofilms formed by three medical relevant species such as *Candida albicans* and two non-*Candida albicans* *Candida* species.

**Methods:** Thirty nosocomial strains were investigated for their ability to adhere and grow in proximity and over body temperature (from 31 to 43°C), mimicking different environmental conditions or severe febrile-like reactions.

**Results:** *Candida* sessile cells reacted to different temperatures showing a strain-specific response. It was observed that the attachment and growth respond differently to the temperature and that mechanism of adhesion has different outputs at high temperature than the growth.

**Conclusions:** This strain-dependent response is probably instrumental to guarantee the best success to cells for the infection, attachment and growth to occur. These observations reinforce the concept of temperature as a major trigger in the evolution of these species especially in this period of increasing environmental temperatures and excessive domestic heating.

## KEYWORDS

biofilm, *Candida*, *Candida albicans*, *Candida* spp., *Candida tropicalis*, temperature

Debora Casagrande Pierantoni and Laura Corte equally contributed to this work. Author order was determined alphabetically.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Mycoses* published by Wiley-VCH GmbH.

## 1 | INTRODUCTION

Microbial biofilms are known to exhibit distinctive characteristics, when compared to free floating cells.<sup>1–4</sup> The majority of bacteria and fungi show the ability to form biofilm, from which they gain peculiar abilities such as a non-genetic driven resistance to drugs and several environmental stressors<sup>5–10</sup> and to higher persistence and lethality.<sup>11,12</sup> The increased tolerance of biofilm can be ascribed to different factors such as the presence of the polymeric extracellular substance,<sup>13,14</sup> an increased activity of the efflux pumps<sup>15–17</sup> or the modulation of the gene regulation.<sup>18</sup> In some cases, the presence of so-called 'persisters', subpopulations of metabolically less active cells tolerant to drugs and other stressing factors, has also been described.<sup>19–21</sup>

Biofilm formation on biotic and abiotic surfaces is finely regulated by genetic factors although many environmental agents, that is oxygen concentration, pH and temperature, play a crucial role in this process. All these aspects have been extensively explored for bacterial biofilms<sup>22–28</sup> while less is known about the behaviour of fungal biofilm, its kinetics or response to stressing factors.<sup>29,30</sup> The lack of such information paved the way to new studies, especially on species like those of the genus *Candida* that are responsible for most of the worldwide fungal infections.<sup>31–34</sup> In fact, the growing population of immunosuppressed patients has resulted in increasingly frequent diagnoses of invasive fungal infections by *Candida* species. *Candida albicans* is the most prevalent and invasive species, frequently isolated in nosocomial environments<sup>35,36</sup> as well as an innocuous inhabitant of the human body.<sup>3,37,38</sup> However, over the last decades, the frequency of infections caused by non-*Candida albicans* *Candida* species (NCAC) has increased,<sup>34,39–41</sup> attracting the interest of researchers also on species such as *Candida tropicalis* and *Candida parapsilosis*, usually associated to natural environments. *Candida parapsilosis*, a yeast normally found in food and natural environments,<sup>42,43</sup> today represents the second or third most frequently isolated *Candida* species from patients, with an increasing incidence in Mediterranean areas and the USA.<sup>44</sup> *Candida tropicalis*, on the other hand, is considered the second most threatful species after *C. albicans*.<sup>45</sup>

The common use of cytotoxic drugs and immunosuppressants together with a scarce, or not fungi-focused hygiene in the medical environment, has risen the frequency of infections caused by *Candida* species able to colonise biotic and abiotic surfaces causing superficial, systemic bloodstream and deep-tissues infections.<sup>13</sup> The ability to form biofilm, in fact, represents one of the most important virulence factors for *Candida* pathogenic species.<sup>38</sup> Moreover, their ability to grow and resist at high temperatures makes these species one of the few fungi capable of surviving at 37°C and even at temperature induced by fever.<sup>46</sup> Thermal tolerance was investigated by Robert et al in relation to fungal virulence, and it was defined as the capacity of fungal species to survive and cause infection at the host temperature.<sup>46</sup> Both the resistance to high temperatures and the ability to form biofilm are threatening factors of these yeast species, the combination of which could have really dangerous outcomes on

the epidemiology of these fungi, especially considering the general increase in average temperatures observed in recent years.<sup>47</sup> To date, the literature about the effect of temperature on biofilm formation of *Candida* species is scarce with few studies carried out on the solely *C. albicans*.<sup>30,48</sup> Therefore, scarce knowledge is available on the ability of *C. albicans* and NCAC species to survive and form biofilm when exposed to the high temperatures implemented by the human body as a natural defence against the infection.

In this frame, we investigated the ability of thirty strains of the species *C. albicans*, *C. tropicalis* and *C. parapsilosis* to adhere and grow at different temperatures, from those close to body temperature to the highest mimics of severe febrile reactions. Two main aspects were considered, the possibility to adhere to a surface and the ability to grow and produce a mature biofilm in 24–48 h, focusing on the possible correlation among the mature biofilm and the number of cells adhered in a short time frame. We tested the hypothesis that both the adhesion step and the development of a mature biofilm would display a large variability associated to different levels of thermal resistance of these strains.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

The 30 strains employed in this study (Table 1) were obtained from the CEMIN Microbial Collection (CMC, CEMIN Excellence Research Centre—University of Perugia). All strains were isolated from nosocomial environments in Italy and afterwards kept frozen at –80°C in 17% glycerol.

Strains identification was carried out by MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) and sequencing of the D1/D2 domain of 26S rDNA subunit (LSU) and Internal Transcribed Spacer (ITS) markers.

All the components of YEPD medium (Yeast Extract 1%, Peptone 1%, dextrose 1%) were purchased from Biolife, while RPMI-1640, crystal violet and menadione were obtained from Sigma Aldrich.

The pre-cultures were obtained by inoculating a loopful of the stock cultures in YEPD medium (7 ml) and were incubated at 37°C for 24 h at 120 rpm. All the tests were carried out with a final cellular density of ca. 10<sup>6</sup> CFU/ml (OD<sub>600</sub> = 0.1). This value was obtained adjusting the pre-cultures density in properly modified RPMI medium, to OD<sub>600</sub> = 0.1 for the biofilm forming ability assay and for the adhesion test.

### 2.2 | Biofilm forming ability test

The strains ability to form biofilm was tested in 96-well microtitre plate by inoculating each strain in two biological replicates, each tested in triplicate. Three different measures of washed biofilms were obtained, at 2, 24 and 48 h of growth, as briefly detailed as follow. 100 µl of standardised cell suspensions was seeded in each

TABLE 1 Strain table

	ID	Species	City of isolation (Hospital)
CA_1	CMC1829	<i>C. albicans</i>	Udine
CA_2	CMC1845	<i>C. albicans</i>	Udine
CA_3*	CMC2000	<i>C. albicans</i>	Pisa
CA_4	CMC1987	<i>C. albicans</i>	Pisa
CA_5*	CMC1968	<i>C. albicans</i>	Pisa
CA_6	CMC1994	<i>C. albicans</i>	Pisa
CA_7	CMC1918	<i>C. albicans</i>	Udine
CA_8	CMC1821	<i>C. albicans</i>	Udine
CA_9	CMC1854	<i>C. albicans</i>	Udine
CA_10	CMC1828	<i>C. albicans</i>	Udine
CA_11	CMC1768	<i>C. albicans</i>	Udine
CA_12*	CMC2026	<i>C. albicans</i>	Pisa
CA_13	CMC2046	<i>C. albicans</i>	Pisa
CA_14	CMC2043	<i>C. albicans</i>	Pisa
CA_15	CMC2008	<i>C. albicans</i>	Pisa
CA_16	CMC2042	<i>C. albicans</i>	Pisa
CA_17*	CMC1959	<i>C. albicans</i>	Udine
CP_1	CMC2039	<i>C. parapsilosis sensu stricto</i>	Pisa
CP_2	CMC1841	<i>C. parapsilosis sensu stricto</i>	Udine
CP_3	CMC1972	<i>C. parapsilosis sensu stricto</i>	Pisa
CP_4*	CMC1981	<i>C. parapsilosis sensu stricto</i>	Pisa
CP_5*	CMC1949	<i>C. parapsilosis sensu stricto</i>	Udine
CP_6*	CMC1951	<i>C. parapsilosis sensu stricto</i>	Udine
CP_7	CMC1973	<i>C. parapsilosis sensu stricto</i>	Pisa
CP_8*	CMC2006	<i>C. parapsilosis sensu stricto</i>	Pisa
CT_1*	CMC1855	<i>C. tropicalis</i>	Udine
CT_2	CMC2040	<i>C. tropicalis</i>	Pisa
CT_3*	CMC1839	<i>C. tropicalis</i>	Udine
CT_4*	CMC2052	<i>C. tropicalis</i>	Pisa
CT_5*	CMC1827	<i>C. tropicalis</i>	Udine

Note: The strains used in this study are listed in the table below, along with source of isolation and the species identification. In the first column, short identification codes are reported.

The starred (\*) strains were used both for the biofilm forming ability test and for the adhesion test.

well, and plates were incubated for 2 h at each selected temperature (31, 34, 37, 40, 41, 42 and 43°C). After the biofilm priming, the inoculum was carefully removed with a multichannel pipette and each well was washed three times with PBS (Phosphate-Buffered Saline).

Washed biofilms were measured by reading the optical density at 405 nm in a plate reader (TECAN Infinite F200). 100 µl of fresh RPMI medium was then added to each well, and plates were incubated 24 h at each selected temperature. After washing, a second measure of washed biofilms was performed. The described procedure was repeated another time to obtain the measure of washed biofilms at 48 h of growth.

### 2.3 | Adhesion test

The two best and the two worst performing strains of each species were selected to test the effect of the temperature on the adhesion step of biofilm growth. Pre-cultures (OD<sub>600</sub> = 0.1) were inoculated in RPMI medium in microtitre plates (96-wells) for a 45 min priming step at 34, 37, 40 and 41°C. After the adhesion step, the wells were washed three times with PBS and fresh RPMI medium was added. The biofilm was incubated around 20 h at each selected temperature in TECAN plate reader (TECAN Infinite F200), measuring the optical density at 405 nm every 5 min.

### 2.4 | Data analysis

Analyses were carried out in R environment (<http://www.R-project.org>) and in MS Excel™, using the editor for VBA programming. Data from the biofilm forming ability test were analysed following the procedure for the determination of low cell-density yeast cultures already developed in our laboratory.<sup>49</sup> Briefly, kinetic parameters such as the lag phase were used to estimate the size of the inoculum, by referring to the density of the cells that were able to adhere to the surface, after the priming step. The determination of the lag phase is based on the most widespread definition that describes it as the transition period during which the specific growth rate increases to that of the exponential phase.<sup>50</sup> The Apparent Lag Phase Extent (ALPE) was calculated on the typical sigmoidal-like growth curve, as the time equivalent at the intersection of the regression curve of the lag phase and that of the exponential phase. The initial cell density (IND) was then calculated taking into account the apparent lag phase extent as described in the equation  $IND = 10^{(\alpha ALPE + \beta)}$ , where  $\alpha$  and  $\beta$  represent, respectively, the angular coefficient and the intercept of the calibration curve obtained in the regression curve of actual cell densities vs ALPE.

## 3 | RESULTS

### 3.1 | Modulation of the biofilm growth by temperature at the species level

The biofilm growth was assessed at different temperatures from 31 to 43°C to test realistic conditions ranging from temperatures easy to find in natural and anthropic environments, including those

occurring in patients with severe febrile status. Biofilm growth was measured after 24 and 48 h by removing the planktonic cells before each reading, to ensure that only the actual biofilm density was measured. Moreover, the removal of free cells after 24 h ensured that the following growth was due only to the cells adherent to the surface. Temperatures between 31 and 40°C were tested with 3°C interval, while the range from 40 to 43°C was tested with a 1°C resolution in order to carefully assess any eventual variations occurring at the higher temperatures, which begin to affect viability.

For *C. albicans*, the 24 h data set showed large variability and even distribution, although at 31°C the strains showed two peaks: a large one without significant growth and a second with little growth (Figure 1A). In the 24 through 48 h time period, there was more growth than during the first day, although some strains remained with little or no growth up to 37°C. Interestingly, the level of the highest growths at 48 h is quite similar at all temperatures, with 31 and 34°C, showing less strains able to grow at high rates.

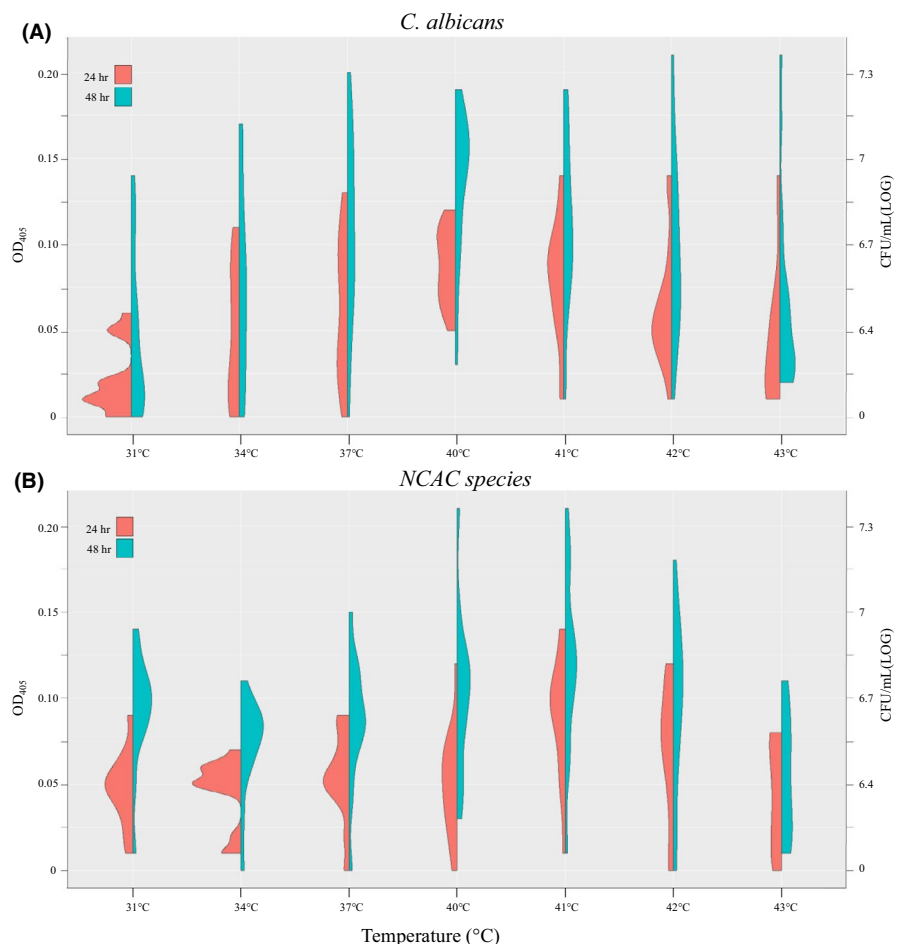
Biofilm formed by *C. parapsilosis* and *Ctropicalis*, after 24 h growth, increased from 31 to 40°C and then decreased at higher temperatures (Figure 1B). Violin plots showed a quasi-normal growth distribution at most temperatures, whereas at 34°C there were two distinct groups of strains, a small one with little if any growth, and a larger with normal growth. The maximum growth was reached between 40 and 41°C, similarly to what observed

in *C. albicans* (Figure 1B). In general, all the strains of this species reached the highest cell densities at 48 h of growth.

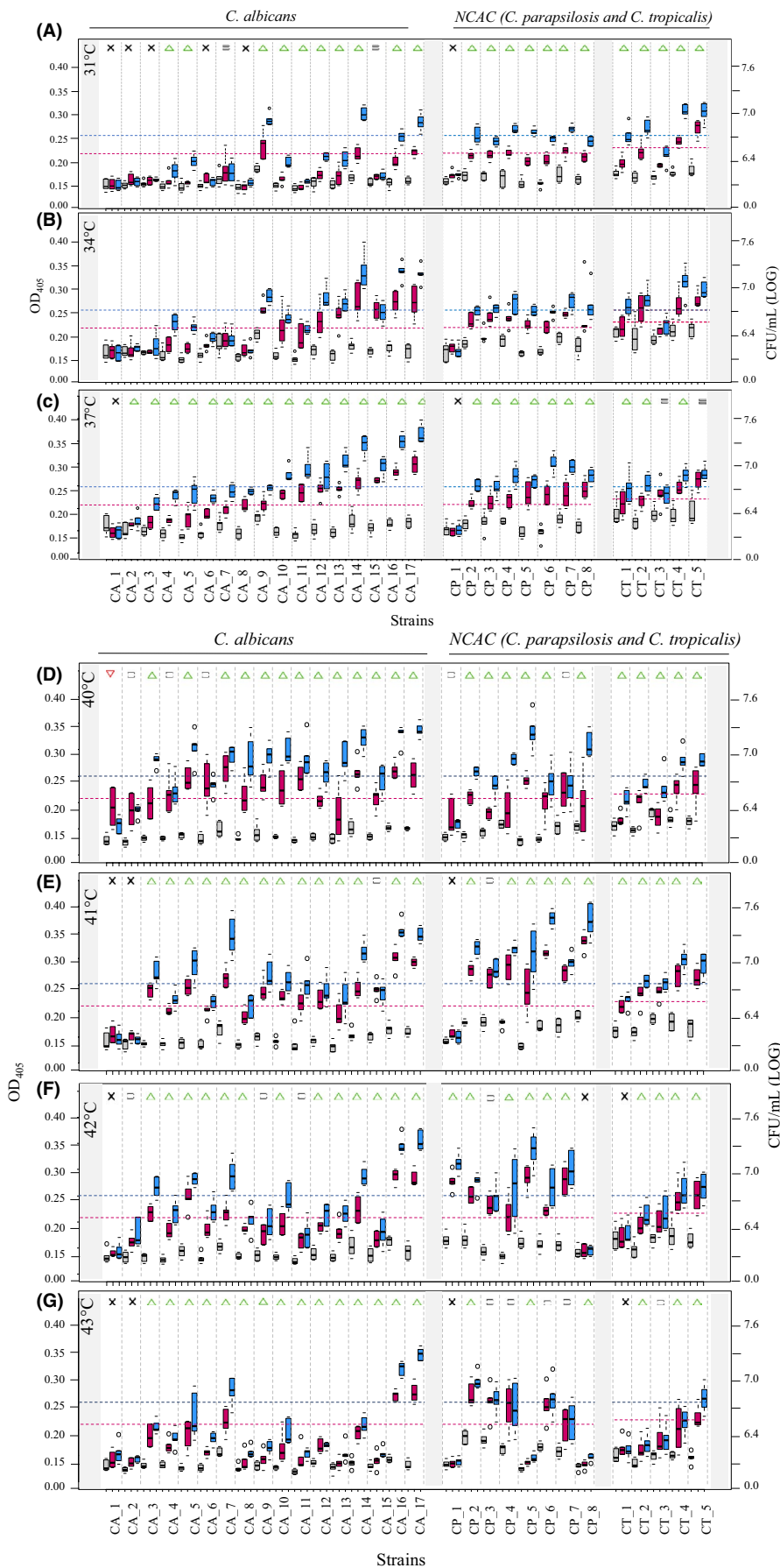
### 3.2 | Strain-specific biofilm production at different temperatures

Throughout these experiments, all species displayed a large variability of the growth spanning from c.a.  $10^6$  to  $5 \times 10^7$  cells (CFU/ml), suggesting to study the distribution of the sessile growth at the single strain level. For this purpose, data from the six technical replicates, derived from the two independent experiments carried out for each tested temperature, were sorted ascendingly to the data of growth at 37°C, to compare the evolution of the biofilm formed by each strain at the different temperatures at 2, 24 and 48 h after the inoculum. Since plates were washed before every reading, the optical density values are referred to the adherent biofilm without the contribution of the planktonic cells produced in the meantime.

*C. albicans* strains grown at 37°C exhibited biofilms that spanned from OD<sub>405</sub> 0.15 to more than 0.35, showing a large variability within the species, although the variability among the replicates was around 5 to 10% (Figure 2C). Whereas few strains did not show a measurable growth within 48 h (CA1 thru CA3), others displayed a constant trend of growth (eg CA5 thru CA9). CA11 grew roughly



**FIGURE 1** Biofilm biomass produced by all the strains at each tested temperature. Violin plots represent the biofilm growth of *Candida albicans* (panel A) and *Candida tropicalis* and *Candida parapsilosis* (panel B) at the selected temperature after 24 h (coral) and 48 h (blue) of growth. Values are blank absorbance (OD<sub>405</sub>) subtracted from the original reading



**FIGURE 2** (A–G) Biofilm biomass produced by the strains at 2, 24 and 48 h at different temperatures. In figure are presented whiskers and box plots summarising the biomass produced by the strains (6 replicates each) at 2, 24 and 48 h, at 31°C (panel A), 34°C (panel B), 37°C (panel C), 40°C (panel D), 41°C (panel E), 42°C (panel F) and 43°C (panel G). In the y-axis are reported the optical density (405 nm) values (left axis) and the respective approximated number of CFU/ml (right axis, logarithmic scale). Grey, magenta and dark-blue boxes indicate the biomass produced, after 2, 24 and 48 h of growth, respectively. In the upper part of the graph of each panel symbols summarised the absence of biomass (X), the biomass increase (up-pointing triangle), decrease (down-pointing triangle) or remain stationary (equal) in the interval 24–48 h. Dark-blue and magenta horizontal dashed lines point the mean value of biomass produced by each species at 37°C at 48 and 24 h, respectively

the same at 24 and 48 h, while strains such as the CA14 showed a greater amount of biofilm biomass at 48 h than at 24 h.

Overall, the NCAC strains have grown less than those of *C. albicans* with the only difference that *C. parapsilosis* isolates displayed some degree of variability with maximum growth varying from  $OD_{405}$  0.15 to ca 0.30, whereas *C. tropicalis* ranged between ca 0.2 and 0.28.

In general, the analysis of the biofilm biomass produced at 37°C revealed that all the strains studied increased their growth during the 2 days of the experiments, as indicated by the green triangle in the upper part of the graph of Figure 2, panel C. Since 37°C is considered the reference temperature for the growth of pathogenic and opportunistic species, the average growth of each species at this temperature was reported as horizontal dashed lines in all panels of Figure 2. Surprisingly, the average growth at 48 h was practically identical throughout the three species ( $OD_{405}$  0.26, 0.25 and 0.25 for *C. albicans*, *C. parapsilosis* and *C. tropicalis*, respectively), while at 24 h, *C. tropicalis* was slightly more performing than the other two species ( $OD_{405}$  0.23 vs 0.21).

Lower temperatures decreased the growth of most strains with few exceptions such as *C. albicans* CA14 and CA17 that displayed very similar behaviour at 37 and 34°C and were over the average growth at all temperatures from 31 to 37°C (Figure 2A–C). The strain CA9 that grew below the species average at 37°C performed comparatively better at lower temperatures for both 24 and 48 h growth. Interestingly, at 34°C, and even more at 31°C, the growth at 2, 24 and 48 h of *C. albicans* was much less variable than at 37°C (Figure 2A–C), whereas at 31°C, the NCAC showed clear differences between the three time points considered.

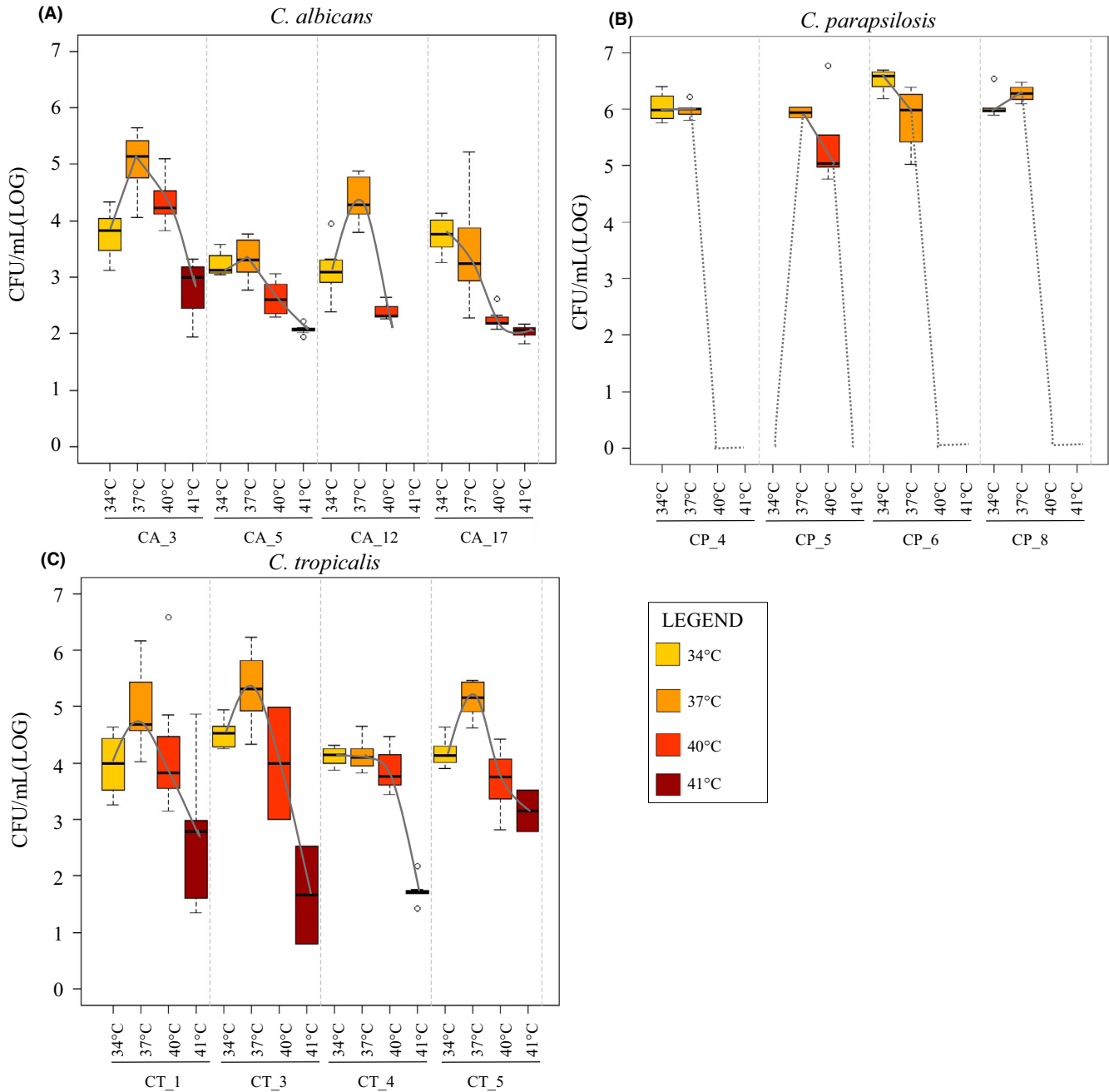
The growth *C. parapsilosis* strains were clustered around the average at all temperatures and growth times, whereas *C. tropicalis* strains CT4 and CT5 grew comparatively better at 34°C and 31°C with values well over the averages, while at 37°C were close to the average. At temperatures over 37°C, the *C. albicans* strains reduced their growth progressively, with exceptions such as CA3, CA5, CA7, CA15, CA16 and CA17 that grew over the average up to 42°C. At 43°C only, the CA7, CA16 and CA17 strains grew more than at 37°C. At 43°C, most of the strains grew poorly with relatively small differences between 24 and 48 h growth (Figure 2D–G). *Candida parapsilosis* strains produced less biofilm biomass over 37°C, except for CP5, CP6, CP7 and CP8 that maintained a good biofilm production up to 42°C whereas at 43°C there was a strong reduction below that at 37°C. The growth reduction observed in *C. tropicalis* was progressive from 40 to 43°C with only CT4 and CT5 maintaining a growth like that at 37°C. Moreover, there was not much difference between the growth at 24 and 48 h indicating that the temperature might have had a devitalising effect during the first hours of growth, or that most of the cells grown during the second day were in planktonic state. In general, *C. tropicalis* strains maintained their relative growth performance within the species, whereas in *C. parapsilosis*, there was a sort of inversion of the growth performance between 37 and 43°C, (Figure 2).

### 3.3 | Effect of the temperature on the adhesion of the biofilm

To test the effect of the temperature on the adhesion of cells to a surface, preliminary experiments were carried out to determine the optimal cell density and time of adhesion, showing that 45 min and  $10^6$  cells per well fell in the optimal range to ensure adhesion. These preliminary tests were necessary to ensure that these two parameters would not play a role as limiting factor to the attachment of cells. Another technical problem encountered was the determination of the cell density at the moment of the adhesion, which is far below the detection limit for both colorimetric and direct optical density assays. For this reason, the estimate of the cells attached in each well was carried out with an approach described elsewhere<sup>49</sup> based on the evidence that there is an inverse linear relation between the density of cells and the extent of the lag phase, thus giving the possibility to calculate with good accuracy the number of cells per well on the basis of the duration of the lag in the growth following the wash out of non-adherent cells.

The results showed relevant differences among the various strains at the tested temperature. Namely, for *C. albicans*, the two strains with poor growth in the previous experiment (hereinafter referred to as bottom-performer in contrast with the two top performer), that is CA3 and CA5 showed different temperature response profiles. In fact, CA3 had a maximum adhesion at 37°C and a steep decrease at higher temperatures, in contrast with CA5 that showed no significant differences in the adhesion at 34 and 37°C and a shallow decrease at higher temperatures (Figure 3A). Strain CA12, one of the top performers in terms of growth, had intermediate adhesion in comparison to CA3 and CA5 and showed maximum adhesion at 37°C and non-detectable adhesion at 41°C. Finally, strain CA17, which grew well at almost all temperatures (Figure 2), had the maximum adhesion at 34°C and then lesser ability to attach to the surface at higher temperatures.

*Candida parapsilosis* strains showed non-detectable adhesion at 40° and 41°C; maximum adhesion at 34°C, with the only exception of CP8, and little differences between 37 and 40°C (Figure 3B). These figures can be explained by the fact that, at the highest tested temperatures, some adhesion occurred, but it was below the detection limit of the lag-time based estimation, around  $10^3$  cells. This hypothesis was confirmed by the fact that the cells could adhere and grow at 40–41°C in the growth experiment, where the cells had the possibility to grow as biofilm for the next 24 and 48 h. In these conditions, the biofilm growth exceeded the detection limit of the direct spectrophotometric reading (Figure 2), indicating that *C. parapsilosis* growth is impeded but not annihilated by high temperatures. Finally, *C. tropicalis* showed a strong difference between the well and the poorly growing strains. The former ones had almost the same pattern (Figure 3C) with maximum adhesion at 37°C, steep decrease from 37 to 41°C and significant less attachment at 34 than at 37°C. A similar pattern was displayed by the well-growing CT5, while the other strain with high growth rate, CT4, had no significant



**FIGURE 3** Estimated number of cells adhered to the surface after a priming step, at four different temperatures of growth (34, 37, 40 and 41°C). The behaviour of all strain belonging to the three different species (*Candida albicans* [panel A], *Candida parapsilosis* [panel B] and *Candida tropicalis* [panel C]), at each selected temperature, is shown in figure. The yellow, orange, red and burgundy boxes indicate the estimated density of the cells adhered to the surface at 34, 37, 40 and 41°C, respectively

differences in the range 34–40°C, followed by a steep reduction of adhesion at 41°C.

#### 4 | DISCUSSION

Biofilm formation is supposed to be the most common form of microbial life in both anthropic and natural environments. Biofilms are responsible for resistance to drugs and stresses and for the

permanence and survival of the microbial species.<sup>1</sup> The ability to attach to a surface under any condition and the vigour of the growth are two of the crucial points for the success of this structure to propagate the strains and maintain its viability. Temperature has been shown to be a primary factor triggering the evolution of pathogenic fungi<sup>47,51,52</sup> especially in this period of increasing average environmental temperatures,<sup>46,47</sup> not to mention the often excessive domestic heating.<sup>53</sup> Less is known about the reaction of fungal biofilm to temperature than other aspects

related to the *C. albicans* genetics involving biofilm formation and growth.<sup>33,54,55</sup> Other species, often referred to as NCAC, are even less studied. Moreover, many studies focus on few model strains, while the studies on freshly isolated strains from different environments are comparatively rare. In this frame, our study focused on the variability among and within species of the reaction of strains to the temperature during the biofilm growth and attachment. The initial hypothesis was that both these two characters have a large variability that could confer different levels of resistance of strains to environmental conditions and even to patients with high fever. As for the growth, all the three species displayed a strain-specific variability at all temperatures and both at 24 and 48 h. Moreover, the strains show different types of reaction to the various temperatures over the 12°C range studied. Few strains were able to grow over the average reference level (37°C) at both 31° and 34°C, as the *C. albicans* CA9 and the *C. tropicalis* CT4 and CT5 strains.

Some strains were able to grow well at practically all temperatures over and below 37°C, leading to the conclusion that the biofilm growth of these microorganisms is partly temperature independent, at least in the range we studied. One such top performer was *C. albicans* CA17 that grew at all temperature over the average growth at 37°C, used as reference, although it has relatively low attachment ability over 37°C. This observation can be extended to all strains of the three species studied and could occur because the attachment befalls in patients at the normal body temperature. From these observations, one can surmise that when the fever occurs, the biofilm is already attached and the only factor for fitness is the ability to withstand temperature and grow. On the other hand, this behaviour would mean that the fever could protect the patient from new colonisations if the fever is present during the infection.

These results show that the ability to form biofilm at temperatures below 37°C, that is close to what is normally considered 'room temperature', can be negligible if any. In turn, this aspect would suggest that the biofilm formation is more related to the temperatures close to the body temperature and would therefore indicate that this character has been strongly influenced by the colonisation of warm-blooded animals in its evolution.<sup>52</sup> In most cases, there was little increase in biofilm formation from 24 to 48 h. This fact could be due to the slight growth of most strains or to the fact that the biofilm is more fragile at these temperatures and therefore easier to be detached by the pre-reading washings. An alternative hypothesis that could not be considered with the settings of this experimental plan is that at higher temperatures, the planktonic cells outgrew the planktonic ones. Attempts to evaluate the amount of removed biofilm were hampered by the scarce quantity and by the interference of the planktonic cells. Biofilm fragility could play a role in cells or patches dispersal, especially in the blood stream, but this mechanical property will likely be better studied with other approaches presently under evaluation in our laboratory.<sup>56,57</sup> The fact that most of the growth was achieved within the first day and might be an explanation of

the figures reporting 20% mortality already at 48 h after the diagnosis and the subsequent vertiginous growth of the mortality in Invasive Fungal Infections (IFI).<sup>58,59</sup> In general, it seems that attachment and growth respond differently to the temperature, which could underline different mechanisms involved. The scarce ability to form biofilm, and to grow as sessile cells, below 37°C could have an evolutionary explanation, in fact, at those temperatures out of the warm-blooded animal bodies, the biofilm could be outperformed by planktonic cells both in terms of growth rate and ease of diffusing in the environment. Once the cells enter the body, the possibility to form biofilm is of paramount importance and 37°C is the optimum temperature for this operation that normally happens in animals without fever. After the biofilm formation, a rapid growth in the range 37–43°C is the only important aspect for the cells to be successful.

Finally, the scarce ability to form biofilm at temperatures over 37°C can be in line with a recent observation that mortality was increased in patients with candidaemia admitted to internal medicine ward without fever with respect to patients with fever.<sup>59–61</sup> These observations could be due to the intrinsic fragility of the patients or to a misidentification of the syndrome in patients without fever that led to the lack of a timely antifungal therapy. In contrast, the absence of fever could be induced by the yeast itself to escape the febrile reaction, as suggested by the fact that biofilm was able to induce the production of anti-inflammatory cytokine IL-10 that reduces phagocytosis and fever induction.<sup>62</sup>

This phenomenon implies that afebrile patients infected with these fungal strains would have more biofilm production and higher risks of mortality. The results of the present study, however, suggest not to generalise these phenomena that could be triggered in a strain-specific manner, inviting to focus on the actual effect induced by specific strains more than on a presumable mean species effect.

A recent paper has evaluated the difference induced in the biofilm transcriptome at 37 and 39°C of *C. albicans* strain, finding that 17 genes were downregulated and 11 upregulated at a significant level.<sup>48</sup> Among the genes involved in cell wall metabolism, *MNN22*, which is repressed in core stress response, was one of the most downregulated genes at 39°C, indicating that this 2°C difference induces an active stress response. Orthologues of this gene are known in different *Candida* species but not in *C. parapsilosis*. The gene *PHO84* is upregulated, whereas *FTH1*, *UCF1* and *PGA45* are repressed when the cell is exposed to antifungal agents and at 39°C, indicating that the temperature increase is somehow comparable to the drug-induced stress. Interestingly, *UCF1* is downregulated in presence of iron, during the transition from yeast to hyphal form and when cells are resistant to fluconazole. Two of these conditions are matched in the case of a biofilm already formed and growing at temperature over 37°C, in fact, there is the presence of iron from the blood and the need to produce more elongated and even hyphal cells. These examples show that there is some correlation between drug dependence and temperature-induced stress, and therefore, the high temperature induces a general response to stress and a metabolic switch. This corroborates our results indicating that the



mechanism of adhesion and growth have different outputs at high temperatures. As a further confirmation of this concept, *PGA26*, encoding for an adhesin-like protein, is downregulated when the temperature increases.

Interestingly, most of the discussed genes have known orthologues in all the *Candida* species investigated in this study, indicating the need of more transcriptome analysis throughout these yeasts at different temperatures.

In conclusion, the *Candida sessile* cells react to the temperature with different strain-dependent responses that are probably instrumental to guarantee the best success to cells for the infection, attachment and growth to occur. These observations reinforce the concept of temperature as a major trigger in the evolution of these species. These three *Candida* spp. differentiated before the whole genome duplication (WGD),<sup>63,64</sup> supposedly dated some 100.000 years ago.<sup>65</sup> Considering that, since 60.000 years ago the temperature grew by some 15°C,<sup>66</sup> the evolution of this characteristic could have been triggered by invasion of modern warm-blooded animals, but facilitated by the external temperature increase.

#### AUTHOR CONTRIBUTIONS

**Debora Casagrande Pierantoni:** Data curation (lead); Investigation (supporting); Visualization (equal); Writing-original draft (lead); Writing-review & editing (supporting). **Laura Corte:** Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal). **Arturo Casadevall:** Conceptualization (supporting); Investigation (supporting). **Vincent Robert:** Conceptualization (supporting); Investigation (supporting); Writing-review & editing (equal). **Gianluigi Cardinali:** Conceptualization (lead); Data curation (supporting); Formal analysis (supporting); Funding acquisition (lead); Investigation (lead); Supervision (equal); Writing-original draft (equal). **Carlo Tascini:** Conceptualization (lead); Investigation (lead); Methodology (supporting).

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### ORCID

Gianluigi Cardinali  <https://orcid.org/0000-0002-4522-7925>

#### REFERENCES

- Watnick P, Kolter R. Biofilm, city of microbes. *J Bacteriol.* 2000;182(10):2675-2679.
- O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Ann Rev Microbiol.* 2000;54(1):49-79.
- Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol.* 2003;11(1):30-36.
- Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. *Eukaryot Cell.* 2005;4(4):633-638.
- Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *The Lancet.* 2001;358(9276):135-138.
- Lewis K. Riddle of biofilm resistance. *Antimicrob Agents Chemother.* 2001;45(4):999-1007.
- Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int J Microbiol.* 2012;2012:1-14.
- Mathé L, Van Dijck P. Recent insights into *Candida albicans* biofilm resistance mechanisms. *Curr Genet.* 2013;59(4):251-264.
- Chatzimochou A, Giampani A, Meis JF, Roilides E. Activities of nine antifungal agents against *Candida auris* biofilms. *Mycoses.* 2020;64(4):381-384.
- Lee CH, Chen YC, Chen IL, Chen FJ, Chien CC. Impact of biofilm production by *Candida* species and antifungal therapy on mortality of patients with candidemia. *Mycoses.* 2020;63(12):1382-1391.
- Corte L, Roscini L, Colabella C, et al. Exploring ecological modelling to investigate factors governing the colonization success in nosocomial environment of *Candida albicans* and other pathogenic yeasts. *Sci Rep.* 2016;6(1):1-9.
- Vitális E, Nagy F, Tóth Z, et al. *Candida* biofilm production is associated with higher mortality in patients with candidaemia. *Mycoses.* 2020;63(4):352-360.
- Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol.* 2006;55(8):999-1008.
- Flemming H-C, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010;8(9):623-633.
- Ramage G, Bachmann S, Patterson TF, Wickes BL, López-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother.* 2002;49(6):973-980.
- De Kievit TR, Parkins MD, Gillis RJ, et al. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother.* 2001;45(6):1761-1770.
- Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence.* 2013;4(3):223-229.
- Goh E-B, Yim G, Tsui W, et al. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci USA.* 2002;99(26):17025-17030.
- Lewis DK. Multidrug tolerance of biofilms and persister cells. In: Romeo T, ed. *Bacterial Biofilms*. Springer; 2008:107-131.
- LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother.* 2006;50(11):3839-3846.
- Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol.* 2007;5(1):48-56.
- Marsh PD. Dental plaque as a biofilm: the significance of pH in health and caries. *Compend Contin Educ Dent. (Jamesburg, NJ: 1995).* 2009;30(2):76-78, 80, 83-87; quiz 88, 90.
- Hošťacká A, Čížnár I, Štefkovičová M. Temperature and pH affect the production of bacterial biofilm. *Folia Microbiol.* 2010;55(1):75-78.
- Gieseke A, Tarre S, Green M, De Beer D. Nitrification in a biofilm at low pH values: role of in situ microenvironments and acid tolerance. *Appl Environ Microbiol.* 2006;72(6):4283-4292.
- Zmantar T, Kouidhi B, Miladi H, Mahdouani K, Bakhrouf A. A microtiter plate assay for *Staphylococcus aureus* biofilm quantification at various pH levels and hydrogen peroxide supplementation. *New Microbiol.* 2010;33(2):137.
- Xu KD, Stewart PS, Xia F, Huang C-T, McFeters GA. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* 1998;64(10):4035-4039.
- Stepanović S, Ćirković I, Mijač V, Švabić-Vlahović M. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. *Food Microbiol.* 2003;20(3):339-343.
- Di Bonaventura G, Piccolomini R, Paludi D, et al. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *J Appl Microbiol.* 2008;104(6):1552-1561.

29. Pettit RK, Repp KK, Hazen KC. Temperature affects the susceptibility of *Cryptococcus neoformans* biofilms to antifungal agents. *Med Mycol*. 2010;48(2):421-426.
30. Pumeesat P, Muangkaew W, Ampawong S, Luplertlop N. *Candida albicans* biofilm development under increased temperature. *New Microbiol*. 2017;40(3):1-14.
31. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of *Candida* biofilm drug resistance. *Future Microbiol*. 2013;8(10):1325-1337.
32. Tsui C, Kong EF, Jabra-Rizk MA. Pathogenesis of *Candida albicans* biofilm. *FEMS Pathogens and Disease*. 2016;74(4):ftw018.
33. Finkel JS, Mitchell AP. Genetic control of *Candida albicans* biofilm development. *Nat Rev Microbiol*. 2011;9(2):109-118.
34. Silva S, Negri M, Henriques M, et al. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev*. 2012;36(2):288-305.
35. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence*. 2013;4(2):119-128.
36. Kim J, Sudbery P. *Candida albicans*, a major human fungal pathogen. *J Microbiol*. 2011;49(2):171-177.
37. Pande K, Chen C, Noble SM. Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. *Nat Genet*. 2013;45(9):1088-1091.
38. Hirota K, Yumoto H, Sapaar B, et al. Pathogenic factors in *Candida* biofilm-related infectious diseases. *J Appl Microbiol*. 2017;122(2):321-330.
39. Abi-Said D, Anaissie E, Uzun O, et al. The epidemiology of hematogenous candidiasis caused by different *Candida* species. *Clin Infect Dis*. 1997;24(6):1122-1128.
40. Kothavade RJ, Kura M, Valand AG, Panthaki M. *Candida tropicalis*: its prevalence, pathogenicity and increasing resistance to fluconazole. *J Med Microbiol*. 2010;59(8):873-880.
41. Miceli MH, Díaz JA, Lee SA. Emerging opportunistic yeast infections. *Lancet Infect Dis*. 2011;11(2):142-151.
42. Rajkowska K, Kunicka-Styczyńska A. Typing and virulence factors of food-borne *Candida* spp. isolates. *Int J Food Microbiol*. 2018;279:57-63.
43. Zupančič J, Babič MN, Gunde-Cimerman N. High incidence of an emerging opportunistic pathogen *Candida parapsilosis* in water-related domestic environments. In: de Loreto, ES, Tondolo, JSM, eds. *Fungal Infection*. IntechOpen; 2018.
44. Tóth R, Nosek J, Mora-Montes HM, et al. *Candida parapsilosis*: from genes to the bedside. *Clin Microbiol Rev*. 2019;32(2):e00111-18.
45. Zuza-Alves DL, Silva-Rocha WP, Chaves GM. An update on *Candida tropicalis* based on basic and clinical approaches. *Front Microbiol*. 2017;8. <https://doi.org/10.3389/fmicb.2017.01927>
46. Robert V, Cardinali G, Casadevall A. Distribution and impact of yeast thermal tolerance permissive for mammalian infection. *BMC Biol*. 2015;13(1):18.
47. Garcia-Solache MA, Casadevall A. Global warming will bring new fungal diseases for mammals. *MBio*. 2010;1(1):e00061-10.
48. Ikezaki S, Cho T, Nagao J-I, et al. Mild heat stress affects on the cell wall structure in *Candida albicans* biofilm. *Med Mycol J*. 2019;60(2):29-37.
49. Casagrande Pierantoni D, Corte L, Roscini L, Cardinali G. High-throughput rapid and inexpensive assay for quantitative determination of low cell-density yeast cultures. *Microorganisms*. 2019;7(2):32.
50. Swinnen I, Bernaerts K, Dens EJ, Geeraerd AH, Van Impe J. Predictive modelling of the microbial lag phase: a review. *Int J Food Microbiol*. 2004;94(2):137-159.
51. Bergman A, Casadevall A. Mammalian endothermy optimally restricts fungi and metabolic costs. *MBio*. 2010;1(5):e00212-10.
52. Robert VA, Casadevall A. Vertebrate endothermy restricts most fungi as potential pathogens. *J Infect Dis*. 2009;200(10):1623-1626.
53. Flannigan B, Samson RA, Miller JD. Microorganisms in home and indoor work environments: diversity, health impacts. *Investigation and Control*. CRC Press; 2003.
54. Araújo D, Henriques M, Silva S. Portrait of *Candida* species biofilm regulatory network genes. *Trends Microbiol*. 2017;25(1):62-75.
55. Nobile CJ, Fox EP, Nett JE, et al. A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell*. 2012;148(1-2):126-138.
56. Mattana S, Cardinali MA, Caponi S, et al. High-contrast Brillouin and Raman micro-spectroscopy for simultaneous mechanical and chemical investigation of microbial biofilms. *Biophys Chem*. 2017;229:123-129.
57. Roscini L, Vassiliou A, Corte L, et al. Yeast biofilm as a bridge between medical and environmental microbiology across different detection techniques. *Infectious diseases and therapy*. 2018;7(1):27-34.
58. Bassetti M, Molinari M, Mussap M, Viscoli C, Righi E. Candidaemia in internal medicine departments: the burden of a rising problem. *Clin Microbiol Infect*. 2013;19(6):E281-E284.
59. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of *Candida* bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob Agents Chemother*. 2005;49(9):3640-3645.
60. Tascini C, Falcone M, Bassetti M, et al. Candidemia in patients with body temperature below 37°C and admitted to internal medicine wards: assessment of risk factors. *Am J Med*. 2016;129(12):1330.e1-1330.e6.
61. Wisplinghoff H, Seifert H, Wenzel R, Edmond M. Inflammatory response and clinical course of adult patients with nosocomial bloodstream infections caused by *Candida* spp. *Clin Microbiol Infect*. 2006;12(2):170-177.
62. Chandra J, McCormick TS, Imamura Y, Mukherjee PK, Ghannoum MA. Interaction of *Candida albicans* with adherent human peripheral blood mononuclear cells increases *C. albicans* biofilm formation and results in differential expression of pro-and anti-inflammatory cytokines. *Infect Immun*. 2007;75(5):2612-2620.
63. Dujon B, Sherman D, Fischer G, et al. Genome evolution in yeasts. *Nature*. 2004;430(6995):35-44.
64. Turner SA, Butler G. The *Candida* pathogenic species complex. *Cold Spring Harb Perspect Med*. 2014;4(9):a019778.
65. Wolfe KH, Shields DC. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*. 1997;387(6634):708-713.
66. Bintanja R, van de Wal RS, Oerlemans J. Modelled atmospheric temperatures and global sea levels over the past million years. *Nature*. 2005;437(7055):125-128.

**How to cite this article:** Casagrande Pierantoni D, Corte L, Casadevall A, Robert V, Cardinali G, Tascini C. How does temperature trigger biofilm adhesion and growth in *Candida albicans* and two non-*Candida albicans* *Candida* species?. *Mycoses*. 2021;64:1412-1421. <https://doi.org/10.1111/myc.13291>