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Original article

The pathogenic and colonization potential of *Candida africana*

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ABSTRACT

The *Candida albicans* population displays high genetic diversity illustrated by 18-well differentiated genetic clusters. Cluster 13, also known as *Candida africana*, is an outlying cluster and includes strains first described as atypical *C. albicans* isolates of vaginal origin, showing apparent tropism for the female genital tract. In our study, we combined *in vitro*, and *in vivo* models to explore the colonization and pathogenic potential of *C. africana*. We report that *C. africana* has similar fitness to *C. albicans* when it comes to colonization of the oral and vaginal mucosa, however it has decreased fitness in gastrointestinal colonization and systemic infection. Interestingly, despite high population homogeneity, our *in vitro* data highlighted for the first time a variability in terms of growth rate, biofilm formation and filamentation properties between *C. africana* strains. Overall, our data lays the foundations for exploring specific features of *C. africana* that might contribute to its apparent niche restriction.

During its evolution, the polymorphic yeast *Candida albicans* adapted to colonize and persist on various mucosal surfaces of the human body. *C. albicans* is both a commensal resident of the human gut, oral mucosa and genital tracts [1–6], as well as an opportunistic pathogen in immunocompromised patients. Infections caused by *C. albicans* range from superficial mucosal infections, such as oropharyngeal candidiasis (OPC) or vulvovaginal candidiasis (VVC), to severe nosocomial bloodstream infections [7,8]. This remarkable adaptation of *C. albicans* to persist and/or infect various body sites relies on several attributes such as genomic and metabolic plasticity, an array of virulence factors, and the ability to evade the host immune system.

Multilocus sequence typing (MLST) revealed that the *C. albicans* population can be divided into at least 18 well differentiated genetic clusters [9–11]. Although these clusters group *C. albicans* strains originating from different sampling sites and geographical locations, strains from one cluster often tend to have the same geographic origin but rarely display phenotypic specificities [12]. Within the *C. albicans* population, the most remarkable cluster is Cluster 13, also known as *Candida africana*. *C. africana* strains were first described in 1995 by Tietz et al. as atypical, chlamydospore-negative *C. albicans* strains of vaginal origin [13]. Based on their unique features, the newly discovered strains were proposed as a novel *Candida* species, namely *C. africana* [14]. However, further phylogenetic analysis highlighting similarities between *C. albicans* and *C. africana* at the genome level supported that *C. africana* belongs to the *C. albicans* species [15,16]. Yet, for the sake of clarity, below we will use *C. africana* for Cluster 13 *C. albicans* isolates and *C. albicans* for non-Cluster 13 *C. albicans* isolates.

Interestingly, despite a worldwide dissemination, the vast majority of the *C. africana* isolates have been collected from the female genital tract [17,18]. Unlike *C. albicans*, *C. africana* isolates

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are unable to utilize specific carbon sources such as N-acetylglucosamine, glucosamine, DL-lactate, and trehalose [14,19]. It has also been reported that, although being germ tube positive, *C. africana* strains grow and produce filaments slower than other *C. albicans* strains [14,19,20]. Romeo et al. [20]. demonstrated that *C. africana* is unable to form biofilms on polyvinyl chloride strips and have decreased ability to adhere to HeLa cells as compared to *Candida dubliniensis* and *C. albicans* [20]. Moreover, *C. africana* display significantly reduced pathogenicity in a *Galleria mellonella* model of systemic infection [19]. A recent study on a population of 35 *C. africana* strains showed reduced fitness in rich medium (YPD) and two simulative media: saliva simulative medium (SSM) as well as vaginal simulative medium (VSM), suggesting that *C. africana* strains do not have a fitness advantage over other *C. albicans* strains in the female genital tract [11].

In this study, we combined *in vivo* and *in vitro* approaches to evaluate a panel of *C. africana* strains, trying to explore the hypothesis proposed previously by Ropars et al. [11], that *C. africana* strains might be restricted to the genital niche by being less fitted to other human body niches, rather than having specific adaptation to the female genital tract. We reported for the first time that two *C. africana* strains were able to colonize the mouse oral cavity. Strikingly, our *in vivo* experiments revealed a decreased ability of *C. africana* strains to colonize the gastrointestinal tract (GIT) and confirmed a significantly decreased pathogenic potential in a systemic infection model. Surprisingly, the VVC model revealed differences between the two tested *C. africana* strains, as illustrated by the differences in the induced host immune response. We further explored *in vitro* phenotypic heterogeneity among a panel of *C. africana* strains in terms of growth rate, filamentation, and biofilm formation. Our data highlighted for the first time notable phenotypic variability across *C. africana* strains, which was somewhat surprising given their homogeneity in terms of genome sequences. Overall, this study broadens our knowledge about the pathogenic potential of *C. africana* and lays the foundations for exploring the specific features of *C. africana* that might contribute to its apparent niche restriction.

1. Materials and methods

1.1. Strains and growth conditions

In this study, we used a set of 35 *C. africana* strains and two selected control *C. albicans* strains: SC5314 (ATCC MYA-2876) – a high-virulent strain commonly used as a reference obtained from a patient with systemic candidiasis [21,22] and 529L (MYA-4901) - a commensal strain previously shown to stably colonize the murine gastrointestinal tract [23], the oral cavity [24,25] and the vagina [26,27] (Supplementary Table 1). For the *in vivo* murine models, we selected the two *C. africana* strains CBS11016 [28] and 8866 [29]. CBS11016 [28] has been previously used in studies investigating *C. africana* and 8866 [29] was selected as it displays the highest level of heterozygosity among the 35 genome-sequenced *C. africana* strains and is currently used to establish a *C. africana* genome assembly in our laboratory.

Overnight cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C with shaking at 180 rpm.

Biofilm assays were performed using RPMI 1640 medium containing 1% of Fetal bovine serum (FBS) at 37 °C, with shaking at 110 rpm [31].

In filamentation assays, cells were cultured in filamentation-inducing conditions in YPD supplemented with 10% FBS at 37 °C with shaking at 180 rpm for 3 h.

1.2. Murine model of GIT colonization

Mice were cared for in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The animal experiments were performed in accordance with European and French regulations. Protocols were approved by the local ethics committee (Institut Pasteur Center Animal Care Committee, protocol number 10.455). Six 9- to 12-weeks old female BALB/c mice (Janvier Labs) were allowed to rest for 1 week before the experiment. They were given drinking water containing 5% sucrose supplemented with 2 mg/ml streptomycin and 0.1 mg/ml gentamycin starting from 4 days before *C. albicans* inoculation and during the entire experiment. Animals were infected by gavage with 5×10^7 *C. albicans* yeast cells in a final volume of 200 μl of sterile phosphate buffered saline – PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7,4) or given 200 μl of sterile PBS alone for the control group. Stool samples collected at the following days: D1, D2, D3, D4, D7, D9, D11 and D14 were weighed and homogenized in 1 ml of sterile water. Serial dilutions (1:0, 1:10, 1:100, 1:1000) were plated for CFU counting on YPD agar supplemented with 1 g of chloramphenicol and 50 mg of gentamycin per 1L. Two independent experiments were performed.

1.3. Murine model of OPC

All mouse experiments were conducted in strict accordance with the guidelines of the Swiss Animals Protection Law and approved by the Veterinary Office of the Canton of Zürich in Switzerland (license numbers 141/2021). Female C57BL/6 mice (Janvier) were housed at the Laboratory Animal Service Center of University of Zürich under Specific-pathogen-free (SPF) conditions. Mice were allowed to rest for 1 week before the experiment. 6–10 weeks old animals were infected sublingually with 2.5×10^6 *Candida* yeast cells as described [30] without immunosuppression. Briefly, mice were anesthetized by intraperitoneal injection with 65 mg/kg Ketamine and 13 mg/kg Xylazine. A 2.5 mg cotton ball soaked in fungal suspension (5×10^7/ml in PBS) was placed under the tongue for 80–90 min. To maintain constant anesthesia during the entire duration of infection, two additional injections of 17.5 mg/kg Ketamine and 3.5 mg/kg Xylazine were administrated 30 min and 50 min after the first injection. The mice were kept on a heating mat at 37 °C during the entire period of anesthesia and injected subcutaneously with 10 ml/kg saline to stabilize the circulation. Vitamin A ointment was applied onto the eyes to avoid drying out of the eyes. The mice were monitored during the entire period of anesthesia and until they had recovered for any signs of distress. To determine the fungal burden, mice were euthanized at the day 1 or day 7 p. i., the tongues were removed, homogenized in sterile 0.05% NP40 in H_2O for 3 min at 25 Hz using a Tissue Lyzer (Qiagen) and serial dilutions were plated on YPD agar containing 100 μg/ml Ampicillin.

Isolation of total RNA from bulk tongues was carried out according to standard protocols using TRI Reagent (Sigma). cDNA was generated by RevertAid reverse transcriptase (Thermo Fisher). Quantitative PCR was performed using SYBR Green (Roche) and a QuantStudio 7 Flex instrument (Life Technology). The oligonucleotides used for qPCR are listed in Supplementary Table 2. All qPCR reactions were done in duplicates and the relative expression of each gene was determined after normalization to the housekeeping gene *Actb*.

Tongue tissue for histological assessment was fixed in 4% PBS-buffered paraformaldehyde overnight and embedded in paraffin. Three sagittal sections (9 μm) per sample were stained with

Periodic-Acid Schiff (PAS) reagent, counterstained with hematoxylin and mounted with Pertex (Biosystem) according to standard protocols from the Laboratory Animals Mouse Pathology Service, University of Zurich. Images were acquired with a digital slide scanner (NanoZoomer 2.0-HT, Hamamatsu) and analyzed with NDP.view2. Images were assessed qualitatively for *C. albicans* morphology and the presence of neutrophils.

1.4. Murine model of VVC

All animal experiments concerning the VVC model were performed in agreement with the EU Directive 2010/63, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the National Italian Law 116/92. The protocol was approved by the Modena and Reggio Emilia University Ethics Committee for animal care and use (Comitato Universitario di Bioetica and Organismo Preposto al Benessere degli Animali, 74/2023-PR). All the animals were housed in the animal facility of the University of Modena and Reggio Emilia (Centro Servizi Stabulario Interdipartimentale, BIOSTAB, Authorization number 268/2011-A), under SPF conditions.

Female CD1 mice (Charles River), were purchased at 16–18 g of weight and allowed to rest for 1 week before the experiment. Mice were maintained under a pseudoestrus condition by subcutaneous injection of 0.2 mg of estradiol valerate in 100 μl of sesame oil (both from Sigma–Aldrich) 3 days prior to infection. The day of infection (day 0) mice were vaginally injected with 10 μl of *Candida* strains, containing 2×10^7 fungal cells or saline (mock infection). To favor vaginal contact and adsorption, mice were held head down for 1 min following inoculation. At day 1 post-infection, the vaginal lumens were thoroughly washed with 250 μl of saline, given in five separate 50 μl volumes. The washes were collected, 10 μl were immediately analyzed for LDH release. Vaginal washes were then centrifuged at 2500 rpm for 5 min and the supernatants were stored at −20 °C before being tested for IL-1β production (see below). The total cellular fractions were resuspended in 200 μl of PBS. 100 μl thereof were used for the flow cytometry analysis and 100 μl were used for the CFU quantification.

Cellular fractions obtained from vaginal washes were stained with: PE-conjugated mAb to mouse Ly-6G (GR-1; 0.05 μg/test; Rat IgG2bk; Thermo Fisher Scientific), APC-conjugated mAb to mouse CD11b and FITC-conjugated mAb to mouse CD45 (both 0.25 μg/test; Rat IgG2bk, BioLegend) for 20 min at RT in the dark. After incubation, cells were washed twice with fluorescence buffer (PBS + 1% FCS + 0,5% NaN3 2M) and resuspended in 0.5 ml of FB. Then, 7AAD (10 μl/test; Thermo Fisher Scientific) was added and incubated for 10 min in the dark at 4 °C. Finally, the samples were analyzed by a FACSDiva™ cytofluorimeter with the FACSDiva software version 6.1.3 (BD, Becton Dickinson). Control isotypes antibodies were also included in the experiments.

The supernatants from vaginal washes were tested for IL-1β by ELISA (MyBiosource). Cytokine titers were calculated relative to standard curves.

For monitoring of the vaginal fungal burden, cellular fraction obtained after vaginal lavage were serial diluted and plated in Sabouraud agar containing chloramphenicol (50 μg/ml) (both from Sigma–Aldrich), incubated for 48 h at 37 °C, and the CFU were counted.

Host cell damage was assessed through evaluation of LDH release by damaged cells using a specific colorimetric kit (LDH Assay Kit (Cytotoxicity), abcam).

1.5. Murine model of systemic infection

Mice were cared for in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The animal experiments were performed in accordance with European and French regulations. Protocols were approved by the ethics committee (Institut Pasteur Center Animal Care Committee, protocol number 10.455). 9- to 12-weeks female BALB/c mice (Janvier Labs) were allowed to rest for 1 week before the experiment. The mice were anesthetized using 5–10 mg/kg of Xylazine and 80–100 mg/kg of Ketamine according to the mice weight, and infected retro-orbitally with 5×10^5 *Candida* cells in 100 μl of sterile water or with 100 μl of sterile water only (control mice). Animal welfare and weight was assessed daily for 11 days. Mice that lost over 20% of their weight were euthanized by CO_2 asphyxiation and both kidneys were isolated. Similarly, to follow the fungal burden progression, mice were sacrificed at D2, D4 and D7 and their kidneys were isolated. The isolated kidneys were weighed and homogenized in 1 ml of sterile water and serial dilutions (1:0, 1:10, 1:100, 1:1000, 1:10,000) were plated for CFU counting on YPD agar supplemented with 1 g of chloramphenicol and 50 mg of gentamycin per 1L. Two independent experiments were performed.

1.6. Doubling-time

The assay was performed in YPD in 96-well plates containing 100 μl of culture of each strain inoculated at an initial OD_{600} of 0.1. Plates were incubated at 30 °C with agitation and the OD_{600} of the cultures was measured every 10 min using a microplate reader (Tecan Sunrise). The doubling time was calculated by dividing by 2 the time between $OD_{600} = 0.15$ and $OD_{600} = 0.6$. The growth curves were performed in three independent experiments.

1.7. Filamentation assay

To assess filamentation, the strains were cultured in 3 ml of YPD supplemented with 10% FBS at 37 °C with shaking at 180 rpm for 3 h in 24-deep-well plates. Cells were observed using an Olympus IX 83 microscope equipped with a 20× or 40× objective. For each strain, five regions were randomly selected, and images were captured with the Hamamatsu ORCA Flash cooled CCD camera, using the Cell Sens software. Filamentation phenotypes were scored quantitatively and qualitatively by eye as “poorly filamentous” to “highly filamentous” (indicated as scores of 1–5 respectively) and as “hyphae”, “pseudohyphae” and “both pseudohyphae and hyphae” (indicated as H, P and P/H, respectively). The assessment of filamentation was performed independently by two persons, the results are presented as a consensus of the two analysis.

1.8. In vitro biofilm formation

The biofilm formation assay was performed as described [31] in 12-well plates in RPMI 1640 containing 1% of FBS. The plates were inoculated with 2 ml of cell suspensions ($OD_{600} = 0.2$ per ml) and incubated at 37 °C without agitation for 90 min for the initial cell adhesion. The non-adherent cells were then washed with 2 ml of PBS and the same volume of fresh medium was added. Plates, sealed with Breathing sealing membranes (Greiner bio-one), were incubated for 24 h with agitation (110 rpm). Further, the medium was carefully aspirated, and biofilms were gently washed with 2 ml of PBS. Biofilms formed at the bottom of each well were scraped

and transferred to pre-weighed nitrocellulose filters. The filters containing biofilm were dried at RT for 48 h and weighed. The average biofilm biomass for each strain was calculated after subtraction of the empty filter weight. The three independent experiments were performed.

1.9. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test or unpaired t-test. The data are represented as mean ± standard error of the mean (SEM). A p-value of <0.05 was considered statistically significant. Significance levels are indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Graphs and statistical analyses were generated using GraphPad Prism 9.5.1 (GraphPad Software, La Jolla, CA, USA).

2. Results

2.1. C. africana *is defective for colonization of the murine gastrointestinal tract*

As described previously [32], stable murine gut colonization by *C. albicans* can be achieved upon administration of antibiotics in drinking water to induce microbiota dysbiosis. Mice were inoculated by gavage with the two *C. africana* strains CBS11016 and 8866, or the *C. albicans* control strains SC5314 and 529L, or with PBS (MOCK). The stools were collected at selected timepoints and the CFU were counted (Fig. 1 A). While all mice were colonized 1-day post-gavage at comparable levels (Fig. 1 B), variations emerged from day 4 post-gavage, with a drop in colonization levels by *C. africana* strains, while colonization levels by *C. albicans* strains remained stable (Fig. 1 B). The drop continued until the end of the experiment at day 14 post-gavage, resulting in a strong decrease in colonization for the *C. africana* strains as compared to the *C. albicans* strains (Fig. 1 B, Supplementary Fig. 1). Additionally, at day 14 post-gavage, we also observed differences between the two *C. africana* strains. While 7/12 mice had cleared CBS11016 by day 14, 8866 could still be detected, at low level, in 11/12 mice, suggesting better colonization properties of 8866. We hypothesized that observed decreased gastrointestinal-tract colonization of *C. africana* strains might be linked to specific conditions of the gut microenvironment, e.g. low pH (3), bile salts, hypoxic conditions or presence of gut microbial metabolites. The data (Supplementary Fig. 2) did not reveal any phenotypic differences between *C. africana* and *C. albicans* when cultured under gut mimicking conditions.

2.2. C. africana *persists in the murine oral cavity*

To investigate *C. africana* capacity to colonize/infect the oral cavity, we infected mice sublingually and followed the progress of the fungal carriage and host immune response (Fig. 2 A). At day 1 p.i., we observed decreased tongue fungal burden for one of the *C. africana* strains CBS11016, while mice infected by both strains CBS11016 and 8866 displayed significantly reduced fecal fungal burden (Fig. 2 B). At day 7 p.i., the fungal burden of the two *C. africana* strains in the tongue was comparable to that observed for strain 529L, whereas strain SC5314 was being cleared from the oral cavity (Fig. 2 C). This correlated with our histology imaging of the tongues at day 1 p.i. revealing that while SC5314 led to strong recruitment of polymorphonuclear leukocyte (PMN), the inflammatory response was weaker with 529L, 8866 and CBS11016. Indeed, 592L and 8866 fungal clusters in the tongue epithelium were accompanied by neutrophils in 35% and 13% of cases, respectively (Fig. 2 D and Supplemental Fig. 3). For strain CBS11016, we did not observe any neutrophil recruitment (Fig. 2 D). In terms of fecal fungal burden, strain 529L showed the highest fungal burden (Fig. 2 C). For all tested cytokine and chemokine transcripts, strain SC5314 induced higher immune response than the other tested strains at day 1 p.i.. While strains 529L and 8866 induced similar levels of cytokine and chemokine gene expression, these levels were significantly lower in mice infected with the strain CBS11016 (Fig. 2 E) possibly consequence of initial lower fungal burden (Fig. 2 B). At day 7 p.i., the level of the immune response against SC5314 was significantly decreased, likely linked to the rapid fungal clearance in comparison to other tested strains. Meanwhile, the cytokine and chemokine gene expression in mice infected with strain 529L and the two *C. africana* strains was comparable (Fig. 2 F) demonstrating that *C. africana* induces inflammation at the same extent as *C. albicans* strain 529L.

2.3. C. africana *in the vaginal niche*

We then set out to investigate whether vaginal immunopathology elicited by *C. africana* differs from *C. albicans* using an established mouse model of VVC (Fig. 3 A). We observed that while all four tested strains exhibited similar colonization of the vagina (Fig. 3 B), differences were observed in terms of PMN recruitment, production of the major NLRP3 inflammasome effector IL-1β and tissue damage. Interestingly, the two *C. africana* strains behave differently, the strain 8866, similarly to SC5314, induced significantly higher PMN recruitment (Fig. 3 C), and appeared to induce higher IL-1β production (Fig. 3 D) and damage (Fig. 3 E) than 529L and the other *C. africana* strain CBS11016. This agrees with our data generated *ex vivo* with the vaginal epithelial cell line A431, where we show that 529L and CBS11016 exhibit similar behavior, while SC5314 shows the highest levels of adhesion, invasion and damage, and 8866 shows intermediate behavior (Supplementary Fig. 4).

2.4. C. africana *strains are avirulent in a murine model of systemic infection*

To assess *C. africana* virulence in the murine model of systemic infection, we followed two parameters: animal mortality and progression of kidney fungal burden (Fig. 4 A). Mortality was observed only in mice infected with the strain SC5314, all mice being dead by day 9 p.i. (Fig. 4 B). Of importance, strain SC5314-infected mice showed symptoms including loss of weight, while the mice infected with 529L and the two *C. africana* strains did not show any symptoms nor weight loss (Supplementary Fig. 5). Significantly reduced kidney fungal burden was observed only in mice infected with the two *C. africana* strains, as compared to the *C. albicans* control strains, with a 4-log decrease in CFU at day 7 p.i. for the *C. africana* strains (Fig. 4 C). Interestingly, although the two *C. albicans* control strains SC5314 and 529L showed similar levels of kidney fungal burden, the outcome of the infection differed greatly between the two strains. While 100% of the mice infected with SC5314 died by day 9 p.i., 100% of the mice infected with 529L survived until the end of the experiment at day 11 p.i.

2.5. In vitro phenotyping

C. africana isolates show low genomic diversity compared to isolates from other genetic clusters of *C. albicans* population [11]. In this respect, results presented above were striking as they suggested that *C. africana* isolates might be more phenotypically heterogeneous than expected. Thus, we performed *in vitro* phenotypic tests on a panel of 35 *C. africana* strains (Cluster 13 – Supplementary Table 1) and two control *C. albicans* strains SC5314 and 529L, to better understand differences between *C. africana* and

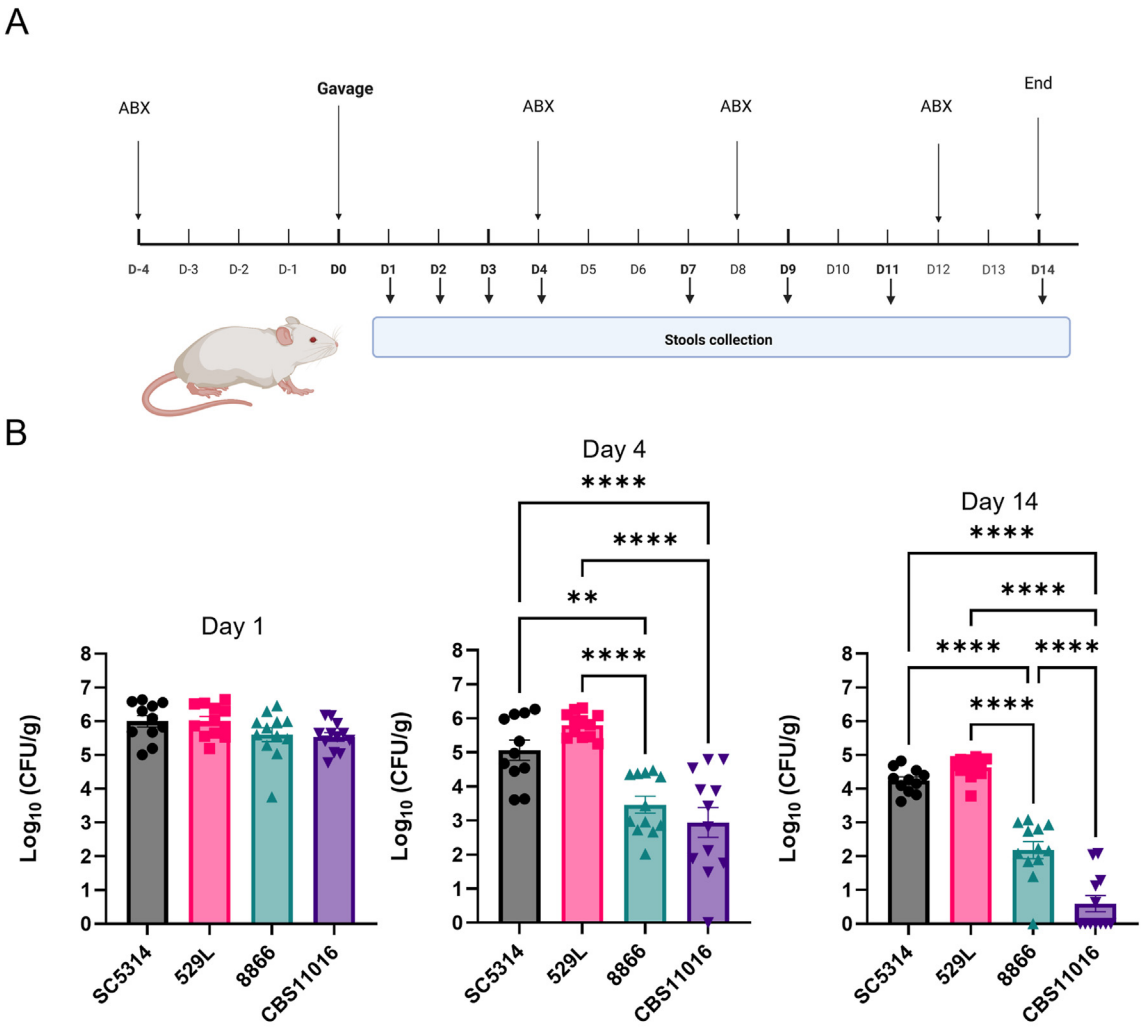


Fig. 1. ***In vivo*** **murine GIT colonization model. A**- Experimental outline. **B-** Comparison of fungal burden in feces of mice inoculated with control strains (529L and SC5314) and two selected *C. africana* strains (CBS11016 and 8866) at day 1, day 4 and day 14 post-gavage. The graphs represent results obtained from two independent experiments. The results are presented as the mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was used in the analysis. Significance levels are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

C. albicans isolates as well as to assess variability within *C. africana* population. Here, we focused on selected *C. albicans* features that are connected to virulence, namely doubling time, biofilm formation and filamentation.

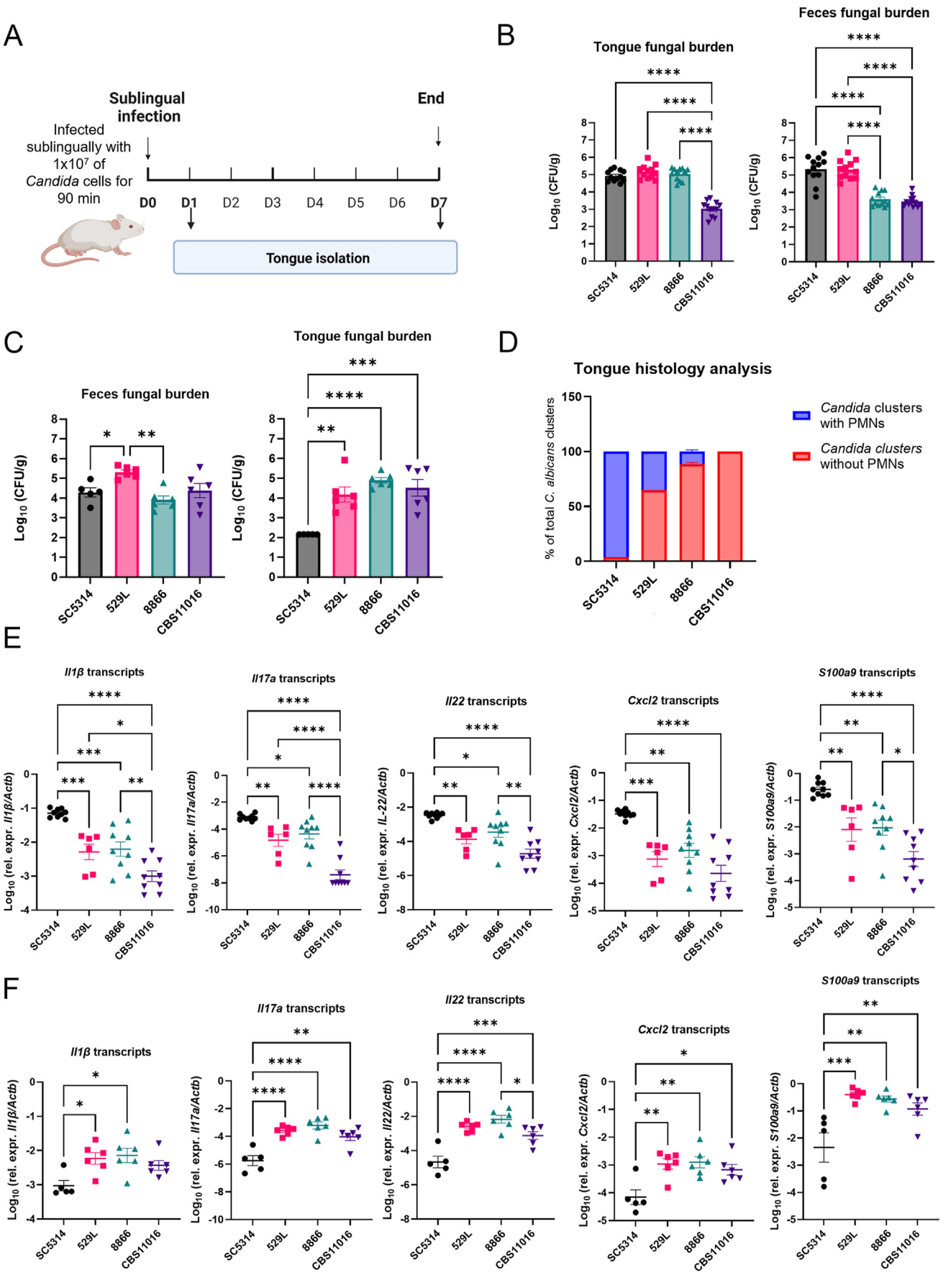
In agreement with previous reports [16,19], we observed that *C. africana* strains have longer doubling times in YPD at 30 °C than other tested *C. albicans* strains (*C. africana* mean = 237 min vs *C. albicans* mean = 95 min (Fig. 5 A). However, the extent of the increase at the population level might be biased by the excessively high doubling time displayed by just a few of the *C. africana* strains, being hyper-filamentous in YPD at 30 °C and forming clumps which surely distorts the OD_{600} reading (Fig. 5 B).

It has been previously reported that *C. africana* strains are unable to form biofilms on polyvinyl chloride strips [20]. Here, we performed biofilm formation tests on polystyrene plates in RPMI 1640 medium containing 1% FBS for 24 h and observed, at the population level, significantly decreased biofilm formation for the *C. africana* strains as compared to the non-*africana C. albicans* strains (Fig. 5 C). However, we noticed variability between *C. africana* strains in their ability to form biofilms with some strains showing similar ability to form biofilms as control strains SC5314 and 529L (Fig. 5 D).

Previous studies investigating *C. africana* filamentation reported that although the strains can filament, they form filaments slower and to a lesser extent than other *C. albicans* strains [19,20]. Here, we observed that *C. africana* strains vary significantly in their ability to form filaments in RPMI 1640 containing 10% FBS upon 3 h of incubation at 37 °C (Fig. 5 E). While some strains are still predominantly found in the yeast form or forming only very short pseudohyphal filaments (XH455, VPCI896/P/12, NCPF 8953), other strains, such as CAAF 1, M1653 or NCPF 8949 form filaments to a similar extent as strain SC5314 (Fig. 5 E).

3. Discussion

Although discovered over 25 years ago, our current knowledge on *C. africana* is still limited. This study is the first thorough investigation of *C. africana* pathogenicity in four different murine models of infection/colonization. The use of different models allowed to highlight differences between *C. albicans* and *C. africana*


A
Sublingual infection
End
Infected sublingually with 1x10⁷ of Candida cells for 90 min
D0
D1
D2
D3
D4
D5
D6
D7
Tongue isolation
B
Tongue fungal burden
Feces fungal burden
Log10 (CFU/g)
SC5314
529L
8866
CBS11016
C
Feces fungal burden
Tongue fungal burden
D
Tongue histology analysis
% of total C. albicans clusters
Candida clusters with PMNs
Candida clusters without PMNs
E
Il1β transcripts
Il17a transcripts
Il22 transcripts
Cxcl2 transcripts
S100a9 transcripts
F
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Il22 transcripts
Cxcl2 transcripts
S100a9 transcripts

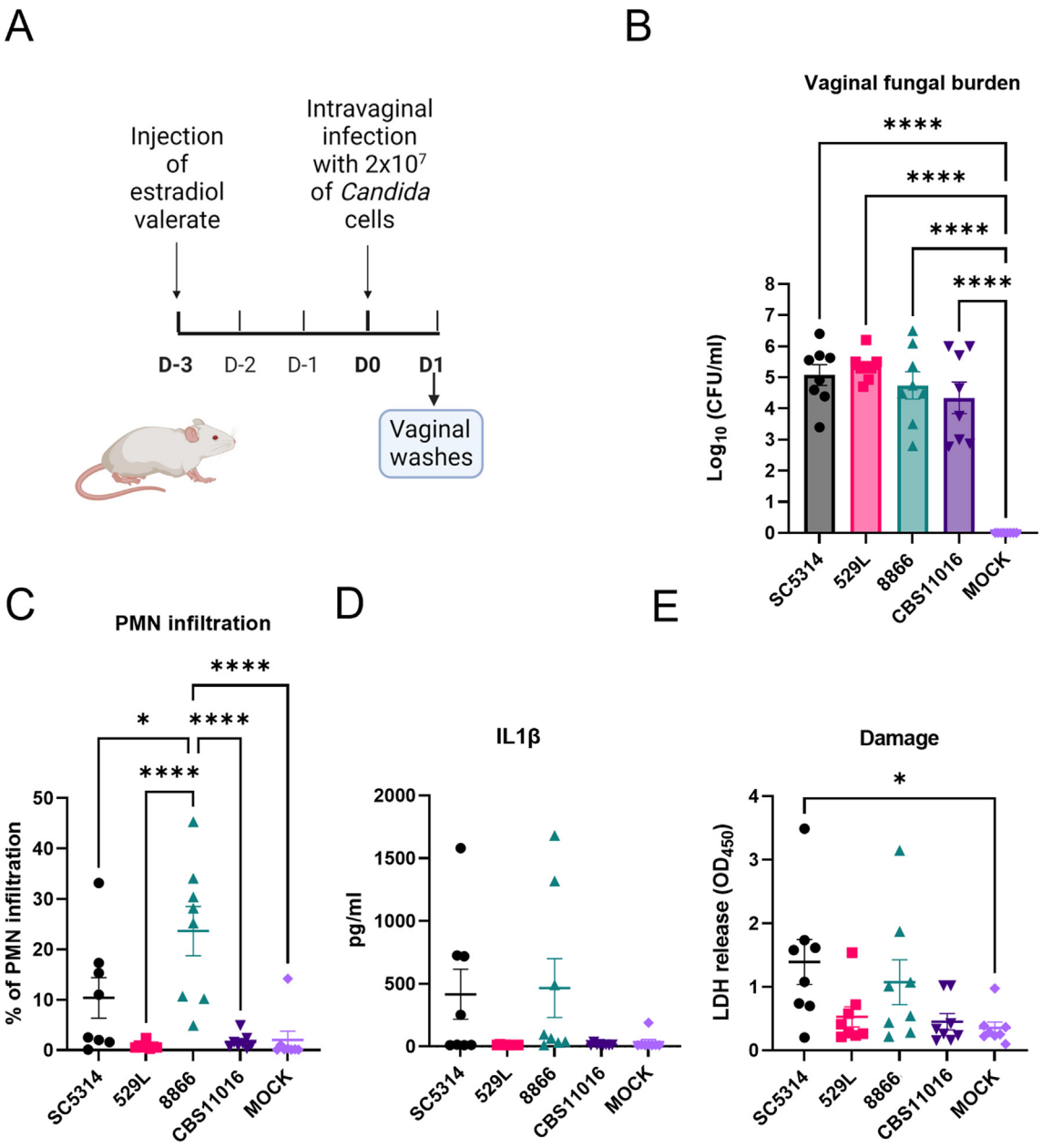

Fig. 3. *In vivo* murine VVC model. A- Experimental outline. **B-** Comparison of vaginal fungal burden in mice inoculated with control strains (529L and SC5314), two selected *C. africana* strains (CBS11016 and 8866) or MOCK infection. **C-** Comparison of PMNs recruitment to the site of infection in mice infected with control strains (529L and SC5314), two selected *C. africana* strains (CBS11016 and 8866) or MOCK infection. **D-** Comparison of IL-1β secreted by mice infected with control strains (529L and SC5314), two selected *C. africana* strains (CBS11016 and 8866) or MOCK infection. **E-** Cell damage induced in mice infected with control strains (529L and SC5314), two selected *C. africana* strains (CBS11016 and 8866) or MOCK infection. The graphs represent results obtained from two independent experiments. The results are presented as the mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was used in the analysis. Significance levels are indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

as well as unsuspected heterogeneity within *C. africana*. The observed phenotypic variability within *C. africana* population was further confirmed *in vitro* analysis on a large panel of *C. africana* strains.

Because *C. africana* shows a tropism for the genital tract [13,17], we set out to compare *C. africana* behavior to *C. albicans* in various mouse models: GIT colonization, OPC, VVC and systemic infection. To the best of our knowledge, there is no report of incidence of *C. africana* in the gastrointestinal tract nor from fecal samples of humans. Our work shows that selected *C. africana* strains are significantly impaired in colonizing the murine gut. We hypothesized that *C. africana* strains might be more susceptible to the

Fig. 2. *In vivo* murine model of OPC. Mice were sublingually infected with two selected *C. africana strains* (CBS11016 and 8866) and two control strains (529L and SC5314). **A-** experimental outline. **B-** Fungal burden in tongue and feces on day 1 post-infection. **C-** Fungal burden in tongue and feces on day 7 post-infection. **D-** Histological assessment of tongues, presented as percentage of neutrophil-containing and neutrophil-less infection foci. **E-** Cytokine and chemokine transcripts in the infected tongue on day 1 post-infection. **F-** Cytokine and chemokine transcripts in the infected tongue on day 7 post-infection. The graphs represent results obtained from two independent experiments. The results are presented as the mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was used in the analysis. Significance levels are indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

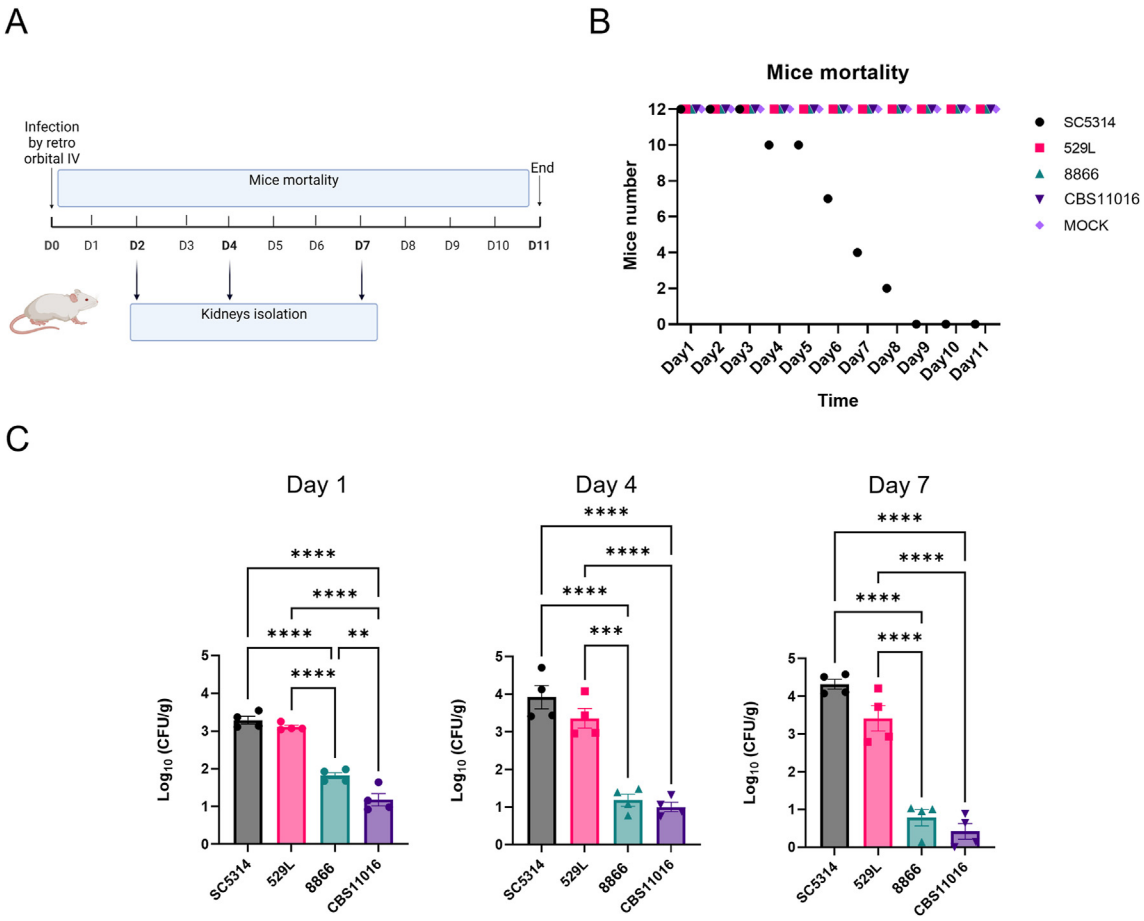

Fig. 4. ***In vivo*** **murine model of systemic infection. A-** Experimental outline. **B-** Comparison mice mortality in mice inoculated with control strains (529L and SC5314) and two selected *C. africana* strains (CBS11016 and 8866) and MOCK. **C-** Comparison of fungal burden in kidneys of mice infected with control strains (529L and SC5314) and two selected *C. africana* strains (CBS11016 and 8866) at day 2, day 4 and day 7 post-infection. The graphs represent results obtained from two independent experiments. The results are presented as the mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was used in the analysis. Significance levels are indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

conditions specific to the gastrointestinal tract environment. However, our data (Supplementary Fig. 3) revealed that *C. africana* strains behave similarly as the *C. albicans* strains SC5314 and 529L when exposed to growth conditions that mimic the gut environment. Deciphering *C. africana*'s inability to colonize the gut will require further studies focusing on peculiar features of these strains. We suspect that one of the nonsense SNPs (single nucleotide polymorphisms) or multiple mutations with synergistic effect, highlighted in the study by Ropars et al. [11], might be responsible for *C. africana* limited potential for gut colonization.

There has been only two reports of *C. africana* presence in the oral cavity, both coming from Iran [33,34]. In agreement with these two reports, our data show that *C. africana* strains can colonize the oral cavity in a similar manner like the commensal *C. albicans* strain of oral origin 529L. In our model, 529L was shown to induce attenuated host immune response and persist longer than the highly virulent strain SC5314 [25]. Based on the observations with the GIT colonization model, we suspect that *C. africana* fungal burden in feces observed at day 7 p.i. do not result from gut colonization but could be explained by cells transiting from the oral cavity through the GIT [35].

The vast majority of *C. africana* strains were isolated from the female genital tract and were associated to vulvovaginal candidiasis [17]. As previously reported [26], our results from the VVC model showed, that the *C. albicans* strain 529L elicited reduced vaginal immunopathology as compared to the *C. albicans* reference strain SC5314. Interestingly, we also observed the most significant differences between the two *C. africana* strains with 8866 inducing significantly higher PMNs recruitment than CBS11016, and 8866 appearing to induce higher IL1β production and cause higher tissue damage. In this regard, 8866 behaves more like SC5314, while CBS11016 is more similar to the commensal strain 529L. Candidalysin has been shown to modulate vaginal immunopathology [26]. Liu et al. showed that the difference between 529L and SC5314 in terms of vaginal immunopathology can be explained by sequence variations at the *ECE1* locus, the candidalysin-encoding gene [26]. Sequence analysis of the 35 *C. africana* genomes did not reveal any *C. africana*-specific *ECE1* sequence variations, suggesting that the differences we observe are not likely to be associated with candidalysin. We suspect that the differences might arise from the variable filamentation potential of the strains, which could be investigated by histological assessment of the mice tissue upon infection with the four strains. Overall, we observe that *C. africana* strains do not appear to have an advantage over other *C. albicans* strains when it comes to colonization of the vaginal mucosa. This was already suggested by Ropars et al. when the authors showed that *C. africana* strains do not exhibit growth advantage in comparison to other *C. albicans* strains when grown *in vitro* in vaginal simulative medium [11].

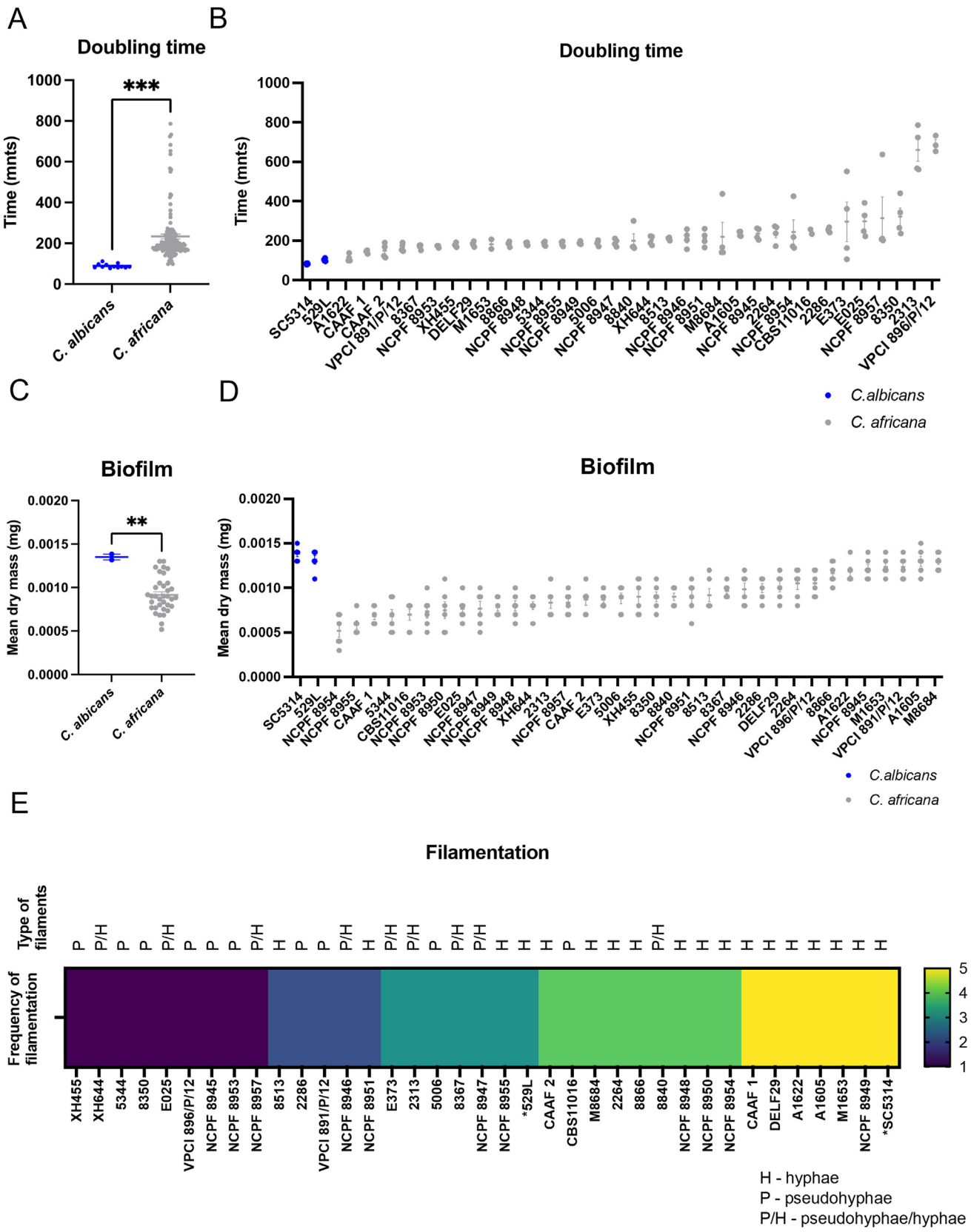

Fig. 5. ***In vivo*** **phenotyping.** **A**- Doubling time comparison between *C. africana* strains and *C. albicans* strains. **B-** Doubling time presented as a mean doubling time for each strain. **C**- Comparison of biofilm formation between *C. africana* strains and *C. albicans non-africana* strains. **D**- Biofilm formation presented as a mean dry mass for each strain. **E-** Filamentation presented as type of produced filaments and filamentation frequency (scale 1–5). *C. albicans* strains are indicated with asterisk (*). The results are presented as the mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test (graphs B and D) or unpaired t-test (graphs A and C) were used in the analysis. Significance levels are indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Two previous studies showed that *C. africana* isolates have significantly decreased pathogenicity in the *G. mellonella* insect systemic infection model [19,36]. Similarly, our results revealed that *C. africana* strains do not cause symptoms and mortality in a murine model of systemic infection, with both *C. africana* strains having significantly decreased kidney fungal burden as compared to the commensal strain 529L and the invasive strain SC5314. Interestingly, we also noticed that although kidney fungal burdens of strains SC5314 and 529L were similar, we did not observe any symptoms nor mortality in mice infected with 529L, when all SC5314 mice had died by day 9. We hypothesize that this might be connected to the filamentation abilities of the two strains. Indeed, 529L is less filamentous than SC5314 [23,25], and it has been previously shown in multiple studies that there is a strong correlation between filamentation and the outcome of systemic infections [37–39].

Finally, specific features of *C. albicans* pathogenicity are its ability to filament and form biofilms. Previous reports showed that although *C. africana* strains are able to produce filaments, they do not filament as readily or robustly than other *C. albicans* strains [13,19]. While we also found *C. africana* strains showing defects in filamentation in response to serum, our work also identified *C. africana* strains which were able to filament to the same extent as *C. albicans* strain SC5314. In contrast to a previous report [20] we showed that *C. africana* isolates can form biofilms in tested conditions, also revealing the substantial variability in terms of biofilm formation within the *C. africana* population.

In conclusion, our study explored for the first time the colonization and pathogenic potential of *C. africana* in various *in vivo* and *in vitro* models. Despite a highly clonal lineage, our work highlights substantial phenotypic variability within the *C. africana* population, a feature that was underestimated until now. Although famous for its tropism to genital tract in patients, we showed for the first time that *C. africana* strains are also able to colonize the oral cavity and induce host immune response in mice. In contrast, we observed that *C. africana* strains have significantly decreased ability to colonize the GIT and invade mice kidneys during systemic infection. Although the molecular mechanisms underlying these phenomena are still unclear and will need further studies, we suspect that this might be linked to the multiple premature STOP codons that are specifically present in the genome of *C. africana* [11]. Overall, our work supports the idea that *C. africana* frequent colonization of the female reproductive tract is not related to an increased fitness in this niche, but rather to a limited ability to colonize other niches, such as the GIT.

Author contribution

D.K., M.L. and C. d. E. designed all experiments except the OPC and VVC murine models. D.K. performed all experiments except the OPC and VVC murine models. D.K., M.L. and C. d. E. analyzed all data except the OPC and VVC murine models. N.S. and M. E. B. performed and analyzed the data for the GIT colonization and systemic infection murine models. R.F.M. and S.L-L. designed, performed and analyzed the data for the murine model of OPC. L.S., A.A. and E.P. designed, performed and analyzed the data for the murine model of VVC. D.K., M.L. and C. d. E. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105230.

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