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Assessment of nanomechanical properties of *Candida albicans* as an element of the oral mycobiota in healthy subjects – a preliminary study

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In a healthy physiological state, the mucous membrane of the oral cavity creates a suitable environment for the colonization of *Candida* spp. yeasts. The aim of the study was to analyze the nanomechanical properties of *C. albicans* cells derived from the oral cavity of healthy people in a biofilm produced in laboratory conditions. *Candida* spp. were sampled from the oral cavity of healthy individuals. The process of biofilm formation was analyzed using classic microscopic observation enriched with SEM (scanning electron microscope) and the nanomechanical properties of the cells were assessed with the use of the atomic force microscopy technique (AFM). From all isolated strains in the samples collected of the oral cavity healthy people was detected 79% *C. albicans*. Other isolated species belonged to the group „non-albicans". The observations of *C. albicans* carried out in 24-h cultures revealed a tendency of the cells to form a biofilm structure

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with multilayer cell systems. The diameter of *C. albicans* cells in this structure was 5.75 µm, and the length of the pseudohyphae was 17.08 µm. The presence of an extracellular substance surrounding the *C. albicans* cells was detected. The mean value of the adhesion force determined for *C. albicans* cells was 4.01 nN. Areas with increased hardness (Force Modulation Mode signal; FMM signal) were found mainly in the zones of cells in contact with the glass substrate. The analysis of *Candida* cells in liquid samples gives satisfactory results, as it prevents unfavorable changes in the cell surface and thus provides more reliable results. The quality of the biofilm is probably related to the nanomechanical properties of *C. albicans* cells and may consequently contribute to the stability of the biofilm structures and their susceptibility or resistance to antifungal drugs.

The presence of *Candida* spp. especially in companion animals (dogs, cats) poses a risk of their transmission to the human organism. For this reason, it is advisable to undertake additional research to analyze the ability of zoonotic-origin *Candida* spp. to form biofilms with comparison of the biofilm-formation capacity of species isolated from humans.

KEY WORDS: yeast / mycobiota / AFM / healthy subjects

The composition of the microbiota of the skin, oral cavity, and gastrointestinal and urogenital tracts varies, as these regions are colonized by a varying number and species composition of bacteria, archaea, bacteriophages, and fungi. The oral cavity is a unique environment for the growth and persistence of many of these microorganisms due to the specific possibility of contact with the external environment. It is characterized by a highly diversified microbial composition, as the bacterial and fungal microbiocenoses of the dental plaque, tongue surface, and saliva may differ substantially between each other. In healthy people, elements of the microbiota and mycobiota exhibit a delicate balance [Nobile *et al.* 2015, Gulati *et al.* 2016, Malinowska *et al.* 2017, Vesty *et al.* 2017, Ruszkowski *et al.* 2020]. Fungi present in the oral cavity of healthy individuals have been most often identified to represent the genera *Candida, Alternaria, Cladosporium, Aspergilllus, Cryptococcus, Fusarium, Auerobasidium, Aureobasidium,* and *Malassezia* [Nobile *et al.* 2015, Ruszkowski *et al.* 2020, Ponde *et al.* 2021, Pristov *et al.* 2019]. Species from the genera *Saccharomyces*, *Penicillium*, *Scopularis*, and *Geotrichum* are important elements of oral mycobiota as well [Malinowska *et al.* 2017].

Candida albicans, i.e. the most frequent representative of the oral mycobiota, can asymptomatically colonize the oral cavity in healthy people. Simultaneously, this opportunistic pathogen can cause infections of varying severity in certain conditions [Sztajer *et al.* 2014, Lohse *et al.* 2018, Pristov *et al.* 2019, Ponde *et al.* 2021]. In addition to *C. albicans*, the other opportunistic species identified in healthy individuals include *C. glabrata, C. tropicalis, C. krusei*, and *C. parapsilosis* [Pristov *et al.* 2019]. Immune disorders associated with infections and cancer diseases, stress, treatment with immunosuppressive drugs and broad-spectrum antibiotics, post-surgical treatment conditions, changes in diet, nutritional deficiencies [Pieczyńska *et al.* 2020, Yeung *et al*. 2021, 2022], and changes in the oral environment (temporary changes in pH, changes in oxygen levels) can cause an imbalance within the microbiome and promote *Candida* proliferation. This, consequently, may lead to development of various fungal infections from mucous membrane infections (candidiasis) to candidemia [Nowak *et al.* 2009, Lohse *et al.* 2018, Pristov *et al.* 2019, Ponde *et al.* 2021].

The ability of microorganisms to form biofilms is their adaptation to colonize specific environmental niches and, from the medical point of view, a virulence factor leading to the development of infection. Biofilms can form on various biotic (oral cavity, respiratory tract, genitourinary tract) and abiotic (dentures, dental implants, catheters) surfaces [Lal *et al.* 2008, Gulati *et al.* 2016, Pristov *et al.* 2019, Jafri *et al.* 2020, Ponde *et al.* 2021]. They are formed by many *Candida* species (C. *glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis*); however, *C. albicans* is the most common biofilm producer in clinical settings [Pristov *et al.* 2019]. These fungi can form mixed biofilms with bacterial microorganisms involved in the development of inflammatory changes in the oral cavity and periodontium, e.g. *Streptococcus* group oralis - *Streptococcus mutans, Streptococcus gordonii, Staphylococcus aureus, Actinomyces viscosus, and Fusobacterium spp.* [Gulati *et al.* 2016]. *Together with S. mutans, C. albicans* can form dental plaque. A synergistic partnership was observed between *S. oralis*, *S. sanguinis*, and *C. albicans*, where the fungus promoted biofilm formation by the streptococci on abiotic surfaces and on the mucosa [Jafri *et al.* 2020]. *Candida* biofilms are a serious clinical problem, as the fungi are resistant to antifungal drugs and environmental factors [Sztajer *et al.* 2014]. They are also a challenge to the host defense mechanism and can be a source of infection spreading through the bloodstream [Sztajer *et al.* 2014, Gulati *et al.* 2016, Lohse *et al.* 2018].

The aim of the study was to analyze the nanomechanical properties of *C. albicans* cells derived from the oral cavity of healthy people in a biofilm produced in laboratory conditions. The process of biofilm formation was analyzed using SEM (scanning electron microscope) observations and the nanomechanical properties of the cells were assessed with the use of the atomic force microscopy technique (AFM).

Material and methods

Origin of samples, identification of *Candida* spp., and determination of the MIC value

Candida spp. were sampled from the oral cavity of healthy people as part of the research projects: "Occurrence of *Candida* spp. fungi in the oral cavity microflora in physiological balance conditions" and "Analysis of the genetic relationship of *Candida* spp. strains found in the oral microbiota of healthy individuals under physiological balance conditions" (consent of the bioethics committee; Pope John Paul II State School of Higher Education in Biała Podlaska No. 9/ 2018 and No.3/2021/current name of the university: John Paul II University of Applied Sciences).

In laboratory conditions, swabs from the buccal surface were taken (once for each person) from adults declaring the absence of oral cavity infections. The cultures were carried out on Sabouraud agar medium with gentamicin and chloramphenicol (Oxoid) at 35°C for 48 h. The strains were preserved until analysis in Viabank™ for long-term storage of microorganisms at low temperatures (Medical Wire & Equipment Co). The strains were then revived in Sabouraud medium (incubation for 24 h, 35°C).

Candida spp. species were identified using the Auxacolor™ 2 kit (Bio-Rad). A 24-hour culture on solid Sabouraud medium with gentamicin and chloramphenicol (Oxoid) was used for the assays. A suspension with McFarland turbidity of 1.5 was transferred with 100 µl of inoculum to each microplate well, sealed with adhesive tape, and incubated at a temperature of 30°C. The readings were carried out every 24 h in accordance with the manufacturer's instructions, and the final interpretation was performed after 72 h.

The MIC values (minimum inhibitory concentration) for fluconazole (FLU), itraconazole (ITC), posaconazole (POS), voriconazole (VO), anidulafungin (AND), micafungin (MYC), and amphotericin B (AMB) against the analyzed *Candida* spp. strains were determined using MTSTM gradient strips and RPMI agar medium (Liofilchem®). In accordance with the manufacturer's instructions, the MIC values were read in 24-h cultures (35°C). The values were referred to the EUCAST guidelines [EUCAST 2023].

C. albicans preparations for light, SEM, and AFM microscopy

50 ml of *C. albicans* culture (35°C; 48h) in Sabouraud liquid medium with dextrose (Oxoid) was centrifuged (8 min 2000 x g). The supernatant was decanted and 30 ml of PBS was added, mixed by inversion, and centrifuged again (8 min 2000 x g). Next, 30 ml of PBS was added to the precipitate, and the suspension was adjusted to 4 McFarland turbidity. Four 1-cm^2 sterile slides were placed on the bottom of a 100-ml sterile container, and 16 ml of the *C. albicans* suspension in PBS was added to obtain a 1-cm layer of the liquid above the slide [Füzik *et al.* 2015]. The samples were incubated to induce adhesion (37°C; 90 min; 75 RPMI rocking shaker), and the slides were rinsed gently by immersion in PBS (10 ml) [Lal *et al.* 2008]. The slides were then placed in liquid Sabouraud medium (16 ml) and incubated at 37°C with 75 RPMI. After 24-h incubation, the slides were removed with sterile tweezers, rinsed three times with PBS (1 ml), and viewed using a light microscope (Nikon, Eclipse Ni-U model; Nikon DS-Fi1c camera; NIS Elements D software; x40 magnification), SEM, and AFM. SEM measurements were performed on a scanning electron microscope Hitachi model TM 3000 equipped with an energy - dispersive X-ray spectroscopy (Oxford Instruments). We used backscattered electron imaging with accelerating voltage of 15kV. *Candida* samples were prepared on 25 x 25 mm quartz coated glass substrates (Ossila, UK) and dried. Then, a thin (fiew nanometers) layer of 99.999% pure copper was sputtered onto them (Testbourne, UK). The Cu layer was deposited with a DC magnetron sputtering system (nano PVD, Moorfield) in an atmosphere of pure argon (99.999%) at a pressure $1{,}8 \cdot 10^{-2}$ mbar.

AFM technique was used to determine in nanoscale local mechanical properties of the samples (based on the force-distance curve) and their 3D topography. Images were collected using a NTEGRA Spectra C microscope (NT-MDT) operating in contact mode (Height, Lateral Force, Error Signal, Force Modulation Mode). In order to provide the samples with physiological conditions, all measurements were

performed in liquid, at room temperature (21°C). All images were acquired using very soft, triangular Si_3N_4 cantilevers designed for imaging of biological samples (MSCT Bruker) with a nominal spring constant of k~0.01 N/m and resonance frequency f_o~7 kHz. Force measurements were conducted by positioning the tip through AFM images over individual *Candida* cells. The data analysis of the 2D surface topographies as well as the 3D representation of the AFM images were performed with the aid of NOVA 1.1.0.1824 software.

The results were analyzed statistically (Statistica v.10 software). The maximum and minimum values, arithmetic average and standard deviations (SD) were calculated.

Results and discussion

The composition of the oral mycobiome varies depending on the analyzed niche. As reported by Vesty, the dental plaque mycobiome was dominated by *Candida* species (>99% of sequences), with the highest proportion of *C. albicans*. In turn, *C. dubliniensis* and *C. tropicalis* were detected less frequently. In saliva, the analysis of sequences revealed the presence of >50% of *Candida*, 6-19% of *Penicillium,* <10% of *Saccharomyces,* and <1% of *Malassezia*; 14-20% of the sequences were not identified to the genus level [Vesty *et al.* 2017]. Other studies of the oral mucosa identified *C. albicans* in 40% of healthy subjects, whereas non-*C. albicans* species were represented by *C. parapsilosis* (15%), *C. tropicalis* (15%), *C. khmerensis*, and *C. metapsilosis* (5%) [Ghannoum *et al.* 2010]. The presence of *C. albicans* was detected in supragingival plaque in 12% of children [Ruszkowski *et al.* 2020] and on the buccal and palate surfaces in 56.5% of healthy adults [Kimsa *et al.* 2020]. The presence of 26 (79%) *C. albicans* strains, 4 (12%) *C. glabrata* strains, 2 (6%) *C. krusei* strains, and 1 (3%) *C. dublinensis* strain was detected in the samples collected from the oral cavity of the healthy subjects. The MIC values of the tested antifungal agents are presented in Table 1. The results were interpreted in accordance with the EUCAST recommendations. All *C. albicans* strains were susceptible to fluconazole (FLU), voriconazole (VO), anidulafungin (AND), micafungin (MYC), and amphotericin B (AMB). Based on the susceptibility to echinocandins, it can be concluded that all strains were also sensitive to caspofungin. Resistance to itraconazole (ITC) and posaconazole (POS) was exhibited by 3 strains (11.5%) and 1 (3.8%) strain, respectively.

All the *C. glabrata* strains were classified as susceptible to the exposure to fluconazole (FLU). The species is characterized by naturally reduced susceptibility to fluconazole, and the present results indicate that the strains do not exhibit acquired fluconazole resistance mechanisms. All the *C. glabrata* strains were sensitive to echinocandins and amphotericin B (AMB). The EUCAST and CLSI recommendations do not provide interpretation of the susceptibility of *C. glabrata* to the other azoles.

C. krusei fungi are naturally resistant to fluconazole (FLU); therefore; regardless of the MIC values, all strains were considered resistant. In the case of this species, the susceptibility to only anidulafungin (AND) and amphotericin B (AMB) can

Table 1. MIC values (mg/L) of the tested *Candida* spp. strains

Species		*C. albicans*	*C. glabrata*	*C. krusei*	*C. dublinensis*
Number of strains tested (N=33)		26 (79%)	4 (12%)	2 (6%)	1 (3%)
Antifungals		MIC			
FLU	min-max	0.19-1	0.75-8	8-96	0.38
	median	0.3	7	52	0.38
ITC	min-max	0.016-0.094	0.094-1.5	0.38-0.5	0.047
	median	0.063	0.75	0.44	0.047
POS	min-max	0.016-0.094	0.064-0.75	0.19-0.25	0.023
	median	0.048	0.625	0.22	0.023
VO	min-max	0.002-0.016	0.032-0.125	0.125-0.25	0.008
	median	0.005	0.094	0.188	0.008
AND	min-max	0.006-0.032	0.047-0.064	0.032-0.064	0.016
	median	0.018	0.064	0.048	0.016
MYC	min-max	0.004-0.008	0.006-0.006	0.064-0.064	0.008
	median	0.006	0.006	0.064	0.008
AMB	min-max	0.19-0.38	0.25-0.5	0.5-1	0.047
	median	0.25	0.38	0.75	0.047

FLU – fluconazole; ITC – itraconazole; POS – posaconazole; VO – voriconazole; AND – anidulafungin; MYC – micafungin; AMB – amphotericin B.

be interpreted using EUCAST. Both strains were sensitive to these antibiotics; additionally, their susceptibility to anidulafungin can evidence their susceptibility to caspofungin. Furthermore, the CLSI guidelines facilitated interpretation of the values for micafungin (MYC) and voriconazole (VO): the analyzed *C. krusei* strains were sensitive to these antibiotics. No interpretation was possible in the case of the other antibiotics.

The *C. dublinensis* strain was sensitive to azoles and amphotericin B (AMB). No interpretation was possible in the case of the other drugs.

Azoles, polyenes, echinocandins, and nucleoside analogs are the main classes of antifungal drugs used in clinical practice. However, biofilms formed by *C. albicans* are often resistant to their action due to the properties of the extracellular matrix, the existence of metabolically inactive "persister" forms, and the upregulation of efflux pumps. These factors hamper effective elimination of *Candida* biofilms [Nobile *et al.* 2015, Gulati *et al.* 2016, Perlin *et al.* 2017, Lohse *et al.* 2018, Gao *et al.* 2018]. Azoles are the largest group of antifungal drugs widely used in clinical practice. However, the resistance of *Candida* to drugs from this group has been reported to increase, hence the increasing importance of echinocandins in the treatment of *Candida* infections [Pristov *et al.* 2019]. Mature *C. albicans* biofilms are highly tolerant to amphotericin B, fluconazole, and caspofungin, which are effective in elimination of planktonic cells [Ponde *et al.* 2021]. One of the components of the extracellular matrix of *C. albicans* biofilms, i.e. the polysaccharide β-1,3-glucan, is involved in fluconazole resistance in this fungus. Hence, the use of β-1,3-glucanase increases the sensitivity of the biofilm to this drug. It is also suggested that β-1,3-glucans present in the biofilm extracellular

matrix can bind specifically to amphotericin B, thereby offering protection to *Candida* cells [Gulati *et al.* 2016]. Species that are unable to produce β-1,3-glucan are sensitive to fluconazole [Ponde *et al.* 2021]. Our study demonstrated that all the *C. albicans* strains were sensitive to fluconazole, voriconazole, anidulafungin, micafungin, and amphotericin B; in turn, 50% of the strains showed resistance to itraconazole and 16.6% were resistant to posaconazole. Since mixed biofilms can be detected in clinical practice, the presence of other *Candida* species in the oral cavity (*C. glabrata*, *C. krusei*, *C. dublinensis*) may affect on effectiveness of elimination such biofilms.

Analysis of *C. albicans* biofilm in light, SEM, and AFM microscopy

As specified in the aim of the study, only *C. albicans* strains were used in further analyses. In laboratory conditions, biofilm formation on a glass substrate was induced for 24 h. The observations of *C. albicans* carried out in 24-h cultures with the use of the light microscope revealed a tendency of the cells to form a biofilm structure with multilayer cell systems. The SEM visualizations showed the formation of biofilm scaffolding and a network of cells and pseudohyphae (Fig. 1/1A-1D). The diameter

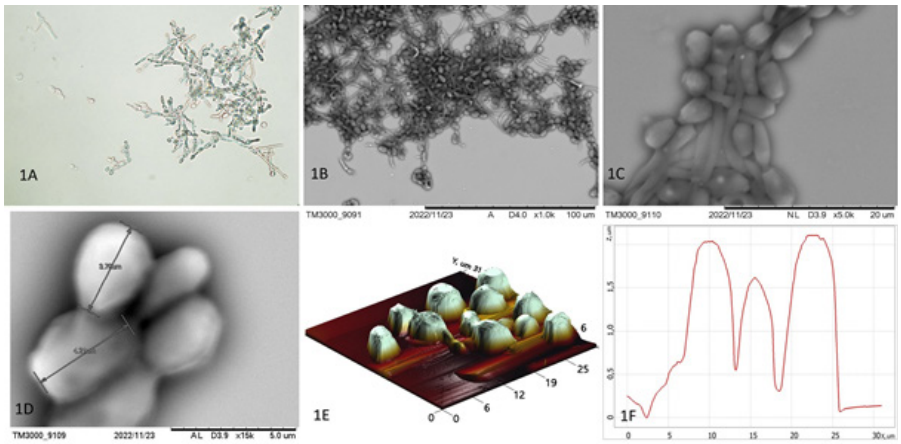

Fig. 1. C. albicans biofilm formation on glass plates in 24-h liquid cultures.

of *C. albicans* cells in this structure was 5.75 µm (min. 3.72 µm, max. 7.73 µm, SD 0.66), and the length of the pseudohyphae was 17.08 µm (min. 6.88 µm, max. 40.40 µm, SD 6.85). The diameters measured with the use of AFM (mean value 4.56 µm, SD 0.84, min. 2.73, max. 6.61) were similar to those obtained in SEM. The height of the cells was measured using the Height Signal in AFM; its average value was 2.84 µm (SD 0.29, min. 1.98 µm, max. 3.35 µm) (Fig. 1/1E-1F; Fig. 2/2A-2B); similar values were reported by El-Baz (2.33 µm) [El-Baz *et al.* 2021] and Tyagi (2.00 µm) [Tyagi *et al.* 2010]. Additionally, the presence of an extracellular substance surrounding the *C. albicans* cells was detected. Figure 2 shows a cell in this structure with a height in the range of 70-200 nm (Fig. 2/2C-2D).

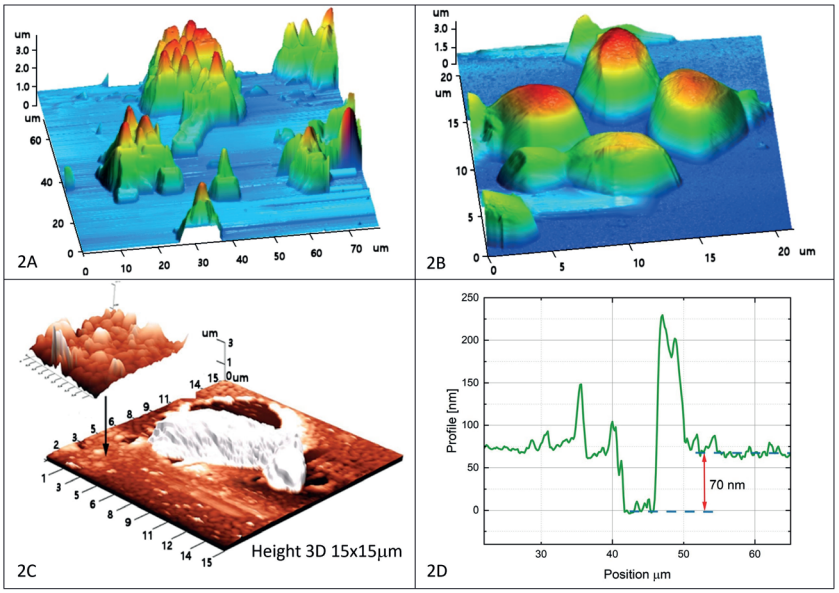

Fig. 2. Topography of C. albicans cells in 24-h cultures on glass with a visible extracellular substance.

The biofilm formation process is influenced by the surface type and environmental factors. The formation of a mature biofilm was observed after 24-48 h on polyvinyl chloride surfaces and after 38-72 h on polymethyl methacrylate surfaces [Ponde *et al.* 2021]. In the present study, the yeast cells were found to adhere to the glass surface after 24 hours and the presence of microcolonies as well as hyphae stabilizing of the structures was detected. Compact, multi-layered cell arrangements were observed in SEM. The biofilm formation ability and virulence of *C. albicans* are associated with the transition from the yeast to hyphal morphotype. Hyphae stabilize the biofilm and have an impact on its layered structure in mature forms, whereas the basal layer plays an important role in the adhesion of the biofilm to the surface. Dispersion of yeast-like cells from the mature biofilm formed on biotic surfaces to other areas in the organism has been reported [Sztajer *et al.* 2014, Gulati *et al.* 2016, Lohse *et al.* 2018, Nobile *et al.* 2015, Ruszkowski *et al.* 2020, Ponde *et al.* 2021]. Therefore, the ability of *C. albicans* to form hyphae is important for the development and maintenance of the biofilm, and strains that do not produce hyphae have an impaired ability to form biofilms [Ponde *et al.* 2021]. The early stage of biofilm formation is influenced by electrostatic interactions determining the process of cell adhesion to biotic or abiotic substrates, van der Waals forces, Brownian movement forces, and hydrophobic interactions. A number of adhesins, e.g. the agglutinin-like sequence (Als) family, the hyphal wall protein (Hwp) family, and others, are expressed in the process. They contribute to effective adhesion of fungal cells to the surface, thereby ensuring biofilm stability [Ponde *et al.* 2021].

AFM analyses of pathogenic microorganisms, including fungi, can be performed using air-dry and liquid preparations. The latter method allows visualization of cells in an environment similar to their natural growth conditions. We employed this type of AFM analysis in the present study. In the liquid environment, the *C. albicans* cell wall is smoother and does not exhibit changes that might be induced by drying, and the cells retain their natural turgor and elasticity [Füzik *et al.* 2015].

The AFM Error Signal (Fig. 3/3B) provides additional qualitative information about the topography of the viewed sample. It facilitates discrimination of small elements against the background of large, relatively smooth surfaces. Areas with different friction coefficients were observed (Lateral Force, LF signal) in the analyzed *C. albicans* samples (Fig. 3/3C). Bright areas with greater friction (rougher) were visible at the sites of contact between the cells and the glass substrate. No bright zones were visible in the lateral and apical cell areas, which proved their lower roughness (greater smoothness). Areas with increased hardness (less elastic; FMM signal) were found mainly in the zones of cells in contact with the glass substrate. The apical areas of the cells, visible as darker zones, were characterized by greater surface elasticity (Fig. 3/3D).

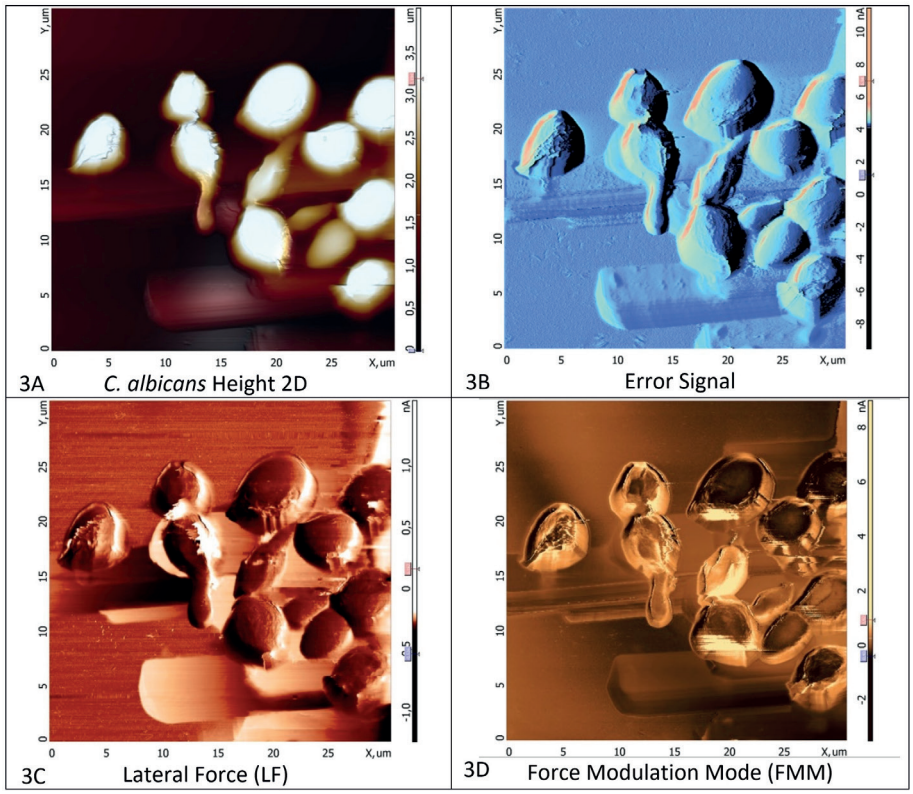

Fig. 3. Topography and nanomechanical properties of C. albicans visualized by AFM (liquid).

Changes in the natural roughness of the surface of *C. albicans* cells and a decrease in their height were observed in AFM upon treatment with essential oils exhibiting antifungal properties (cinnamon oil - MIC 64-500 µg/ml, clove oil - MIC 64-2000 µg/ml, jasmine and rosemary oils - MIC 16-2000 µg/ml). The exposure to clove, cinnamon, jasmine, and rosemary oils resulted in a decrease in the cell height to 381±80 nm, 438±80 nm, 117±25 nm, and 1287±190 nm, respectively, versus the height of 2332±450 nm of control cells. The study conducted by El-Baz also showed that the adhesive capacity of cells increased with the increasing roughness of their surface [El-Baz *et al.* 2021].

The adhesion forces (F_a) determined for the *C. albicans* cells had the same order of magnitude in each case, while the shape of individual curves differed from each other. This suggests gradual detachment of the microscope tip from the sample (cell) surface and indicates "individual" features of the surface of cells of the biofilm-

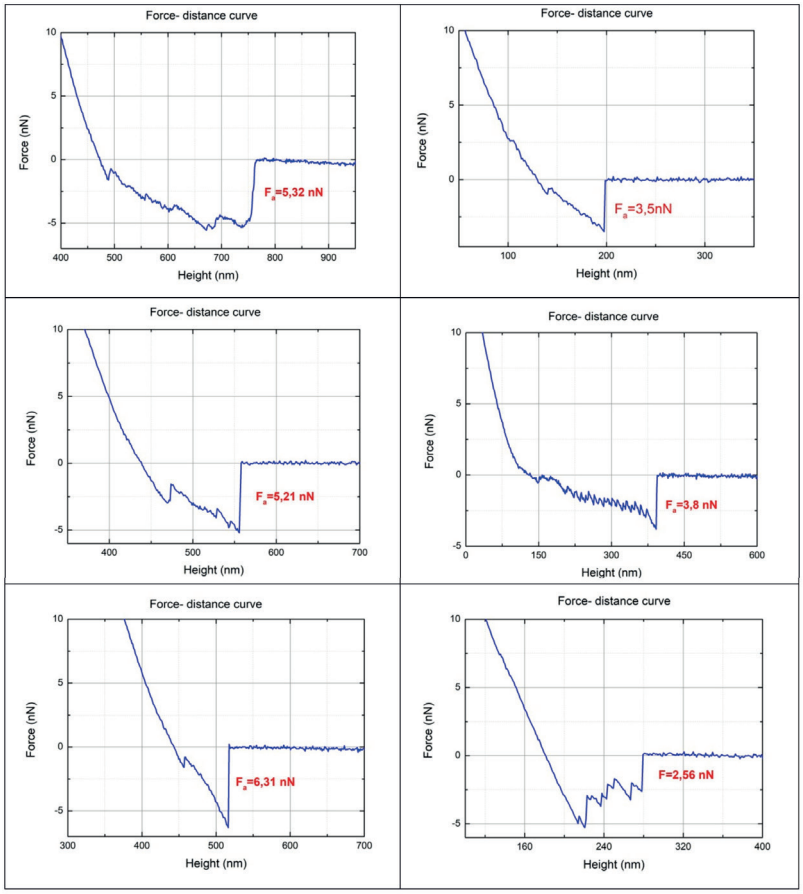

Fig. 4. Adhesion forces (Fa) determined for biofilm-forming C. albicans cells in 24-h cultures.

forming microcolonies (Fig. 4). The mean value of the adhesion force (F_a) determined for *C. albicans* cells present in the biofilm structure after 24-h culture was 4.01 ± 1.64 nN (min. 2.56 nN, max. 6.3 nN). The nanomechanical properties of cells change under the influence of antifungal substances, although they may also be associated with the characteristics of a given strain. Wild-type *Candida* cells were reported to have greater surface elasticity than cells of two mutants (*cho1* Δ/Δ; *kre5* Δ/Δ) or caspofungin-treated cells [Hasim *et al.* 2017]. The adhesion force (force sufficient to detach the AFM tip from the cell surface) may indicate biofilm stability and damage to the biofilm caused by such degrading factors as antifungal agents. The average value of the adhesion force determined in the present study for *C. albicans* cells forming the biofilm structure in the 24-h cultures was 4.01±1.64 nN. In a study conducted by Ma et al., the adhesion force of *C. albicans* cells was 7.35±0.77 nN; after *Streptococcus sanguinis* bacteriocin treatment, it decreased to 5.69±0.62 nN [Ma *et al.* 2017].

In air-dry preparations analyzed in AFM, it is not possible to visualize the extracellular matrix that binds cells constituting the biofilm. This structure, which is extremely important for biofilm stability, can only be observed in liquid samples. The presence of the extracellular matrix around the *C. albicans* cells was shown by AFM in the 24-h cultures. The extracellular matrix visible in AFM binds neighboring cells, and its direct contact with the biofilm substrate has an impact on adhesion forces. Our observations are consistent with other published data showing that extracellular polysaccharides (EPS) are involved in processes of cell adhesion to the surface where biofilm structures are formed and ensure biofilm stability [Algburi *et al.* 2017, Le *et al.* 2022]. An AFM study conducted by Lal et al. demonstrated the presence of EPS in *C. albicans* biofilms [Lal *et al.* 2008]. It has been proved that EPS in planktonic *C. albicans* cells contains mainly carbohydrates, proteins, hexosamine, and phosphorus, while the EPS composition in *C. albicans* biofilm structures has significantly lower amounts of carbohydrates and proteins. The differences in the composition of matrix EPS exert an effect on the process of diffusion of antifungal drugs into the biofilm structure [Lal *et al.* 2008]. The extracellular matrix protects microorganisms in single-species and mixed biofilms against mechanical stresses, effects of environmental factors, and elements of the host immune defense [Ponde *et al.* 2021]. The presence of glucans in the extracellular matrix may inhibit the activation of neutrophils, and thus protect *Candida* cells against host defense mechanisms. In turn, hyphae are able to damage phagocytic cells mechanically and penetrate epithelia during invasion [Nobile *et al.* 2015, Gulati *et al.* 2016]. Natural products can also interact with biofilms. The study conducted by Ansari et al. in AFM and SEM showed that Jujube (*Zizyphus spina-christi)* honey affects biofilms by decreasing the size of mature biofilms and by disruption of their structure. This type of honey affected the cellular morphology of *C. albicans* [Ansari *et al.* 2013]. The antimicrobial peptide (AMP) Psd1 isolated from *Pisum sativum* seeds degraded biofilms and planktonic cell *C. albicans*. Surface alterations, with membrane disruption and leakage of cellular contents, were observed [Gonçalves *et al.* 2017].

Candida spp. infections may also affect warm-blooded animals, e.g. poultry, horses, cows, dogs, and cats. *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* as well as *C. guilliermondii* and other [Seyedmousavi *et al.* 2018, Różański *et al.* 2019]. *Candida* spp. species are isolated from these animals most often. Many of these species are potentially pathogenic to humans, especially immunocompromised patients, the elderly, and subjects with chronic health problems. The presence of *Candida* spp. especially in companion animals (dogs, cats) poses a risk of their transmission to the human organism. Additionally, the increased use of antifungals in susceptible patients may lead to a higher incidence of candidiasis, especially caused by rare species (e.g. *Candida bovina*) [Brunet *et al.* 2020]. For this reason, it is advisable to undertake additional research to analyze the ability of zoonotic-origin *Candida* spp. to form biofilms with comparison of the biofilm-formation capacity of species isolated from humans.

Conclusions

In conclusion, yeasts as opportunistic pathogens can cause endogenous infections in certain conditions. The first stage involves colonization of the surface and biofilm formation, and the key colonization processes take place within 24 h. This is evidenced by the arrangement of cells and pseudohyphae in compact structures visible in SEM. The extracellular matrix visualized by AFM binds neighboring cells, and its direct contact with the biofilm substrate has an impact on adhesion forces. The nanomechanical properties of cell walls, e.g. stiffness, roughness and, consequently, susceptibility to deformation and perforation, as well as adhesion forces may have an impact on the quality of the biofilm.

The analysis of *Candida* cells in liquid samples gives satisfactory results, as it prevents unfavorable changes in the cell surface and thus provides more reliable results. The quality of the biofilm is probably related to the nanomechanical properties of *C. albicans* cells and may consequently contribute to the stability of the biofilm structures and their susceptibility or resistance to antifungal drugs. This requires further analyses on a larger number of strains with comparison of such parameters as elasticity, roughness, and adhesion force with consideration of the impact of antifungal substances on the biofilm formation process and on mature biofilms.

Conflict of interest

The authors declare no conflicts of interest.

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