Sinopfbd, 2023; 8(1): 1-18 **https://doi.org/10.33484/sinopfbd.1310745**

Sinop Üniversitesi Fen Bilimleri Dergisi
Sinop Uni J Nat Sci

ISSN: 2536-4383
E-ISSN: 2564-7873

https://dergipark.org.tr/tr/pub/sinopfbd

In Vitro Evaluation of Biofilm Forming Capacity and Antifungal Resistance of Yeast Isolated from Individuals Aged 18-25 Years

Gülçin ÖZCAN ATEŞ[1] and Müşerref OTKUN[2]

How to cite: Özcan Ateş, G., & Otkun, M. (2023). In vitro evaluation of biofilm forming capacity and antifungal resistance of yeast isolated from individuals aged 18-25 years. *Sinop Üniversitesi Fen Bilimleri Dergisi, 8*(1), 1-18. https://doi.org/10.33484/sinopfbd.1310745

Research Article

Corresponding Author
Gülçin ÖZCAN ATEŞ
gulcinozcan@comu.edu.tr

ORCID of the Authors
G.Ö.A: 0000-0002-8467-2378
M.O: 0000-0001-8977-1140

Received: 06.06.2023
Accepted: 20.06.2023

Abstract

Microbiota studies are an increasingly important issue today. In the literature, there are limited studies on the antifungal resistance and biofilm formation capacity of yeasts isolated from the mouths of young individuals. For this reason, our study was carried out with 133 yeast isolates isolated from the mouths of 17 young individuals between the ages of 18-25 in 2018-2020. When the biofilm-forming capacities of 133 isolates were examined, it was determined that 99.25% were biofilm producers by tissue culture plate method and 66.92% by tube method. One hundred thirty-three yeast isolates and seven reference strains were first evaluated against fluconazole antifungal by agar disc diffusion method. The isolates were found to be susceptible to fluconazole. According to this result, 20 isolates with strong biofilm-forming capacity were selected from 133 yeast isolates. Antifungal resistance was evaluated with fluconazole, itraconazole, clotrimazole, amphotericin B and nystatin gradient test strips. It was determined that 20 isolates were resistant to amphotericin B, and 18 were resistant to nystatin antifungal. It was determined that the sensitivity of itraconazole to 14 isolates and clotrimazole to 3 isolates was dose-dependent. As a result, azole group antifungals can be used mainly in treating oral yeast infections.

Keywords: Antifungal resistance, biofilm, *Candida*, gradient test, oral yeast

18-25 Yaş Arası Bireylerden İzole Edilen Mayaların Biyofilm Oluşturma Kapasitesinin ve Antifungal Direncinin in Vitro Olarak Değerlendirilmesi

[1]Çanakkale Onsekiz Mart University, Vocational School of Health Services, Department of Medical Services and Techniques, Çanakkale/Turkey

[2]Çanakkale Onsekiz Mart University, Faculty of Medicine, Department of Medical Microbiology, Çanakkale/Turkey (Retired)

Öz

Mikrobiyota çalışmaları günümüzde önemi giderek artan bir konudur. Literatürde genç bireylerin ağızlarından izole edilen mayaların antifungal direnci ve biyofilm oluşturma kapasitesi üzerine sınırlı sayıda çalışma bulunmaktadır. Bu nedenle çalışma 2018-2020 yıllarında 18-25 yaş arası 17 genç bireyin ağzından izole edilen 133 maya izolatı ile gerçekleştirilmiştir. 133 izolatın biyofilm oluşturma kapasiteleri incelendiğinde doku kültürü plak yöntemi ile %99.25'inin, tüp yöntemi ile %66.92'sinin biyofilm oluşturduğu belirlendi. 133 maya izolatı ve 7 referans suş ilk önce agar disk difüzyon yöntemiyle flukonazol antifungaline karşı değerlendirildi. İzolatların flukonazole duyarlı olduğu bulundu. Bu sonuca göre 133 maya izolatı arasından biyofilm oluşturma kapasitesi güçlü 20 izolat antifungal dirençliliğin belirlenmesi için seçilmiştir. Antifungal direnç flukonazol, itrakonazol, klotrimazol, amfoterisin B ve nistatin gradyan test şeritleri ile değerlendirilmiştir. 20

izolatın amfoterisin B'ye, 18 izolatın ise nistatin antifungaline karşı dirençli olduğu belirlenmiştir. İtrakonazolün 14 izolata ve klotrimazolün 3 izolata duyarlılığının doza bağımlı olduğu tespit edilmiştir. Sonuç olarak, azol grubu antifungaller ağırlıklı olarak oral maya enfeksiyonlarının tedavisinde kullanılabilir.

Anahtar Kelimeler: Antifungal direnç, biyofilm, *Candida*, gradiyent test, oral maya.

Introduction

Fungi constitute a small part of the oral microbiota; some species cause dental caries, periodontal diseases, and endodontic diseases in the mouth [1-3]. Approximately 75% of healthy individuals carry yeast species as a natural commensal of the oral microbiota. However, yeasts of the genus *Candida* are an opportunistic pathogen that can cause acute or chronic infection in some individuals, especially under various conditions that compromise host immunity. Candidiasis is the most common form of oral disease caused by yeasts of the genus *Candida* [1-4]. *C. albicans* is the most common and pathogenic *Candida* species and has been defined as the most common yeast in oral candidiasis. However, other non-albicans *Candida* (NCAC) species may also contribute to the development of oral candidiasis [4]. It has been determined that oral candidiasis has increased recently due to various factors such as age, prosthetic use, diabetes, cell-mediated immunodeficiency, systemic steroid and antibiotic use, pernicious anemia, malignancy, and head and neck radiation therapy. Therefore, the sensitivity of the oral mycobiota to the antifungal drugs used in treating these diseases is important. Antifungal agents such as polyenes (amphotericin B and nystatin) and azoles (fluconazole, itraconazole, and miconazole) are used to treat oral candidiasis. Azole group antifungals are used in oral applications to treat these diseases because they are mostly inexpensive and non-toxic. Although the resistance of fungi to polyenes is rare, they can gain resistance to the azole group [1-3]. In addition, there is information about increased antifungal resistance due to excessive or unconscious use of antifungal agents. Therefore, antifungal susceptibility testing is needed for an effective treatment of oral candidiasis [1]. The mouth provides an ideal incubation area with the temperature, humidity and nutritive environment required for the reproduction and proliferation of microorganisms. Dynamic complex interactions between host and diet lead to the colonization of microorganisms in the mouth and subsequent biofilm formation. Biofilm formed on teeth or dental materials surfaces has been determined as a virulence factor in forming many oral infections, especially dental caries, endodontic and periodontitis [5]. Pathogenic microorganisms have developed techniques such as colonization, invasion, and pathogenesis, which will contribute to their virulence, many of which are for survival and species continuity. Many virulence factors, such as hemolysis, extracellular hydrolase production, phenotypic change, and adhesion, affect the pathogenesis of *Candida* species. In addition, they produce large amounts of sticky substances in glucose-containing environments. Biofilm formation is also an important factor contributing to the virulence of *Candida* species. Biofilms of *Candida* species are often found on biomaterial implants, the host surface and in

normal sites where they can more easily help escape host defense. They cause persistent infections as they escape the host defense mechanism. Its extensive biofilm-forming ability on catheters and other prosthetic devices contributes to its prevalence as an etiologic agent of intravascular nosocomial infection. Antifungal-resistant *Candida* species producing biofilms pose a major challenge for healthcare professionals and pharmaceutical companies, especially in designing therapeutic and prophylactic strategies. In addition, it causes economic losses due to failure in treatment, high mortality and long-term hospital stay [6-9]. Biofilm formation provides various advantages to microorganisms, such as environmental protection, nutrient availability, metabolic cooperation, and the acquisition of new properties [6-9]. Most importantly, biofilm formation protects microorganisms against natural immunological defences and causes resistance to antimicrobial drugs [10, 11]. Depending on limited nutrients, slow growth and stress conditions, the regulation of cell metabolism and cell density of microorganisms change. This contributes to biofilm resistance. These properties are very important as it is estimated that approximately 65% of human infections caused by microorganisms contain biofilm [11, 12]. Additionally, biofilm-producing species have been associated with the death of infected patients [11, 13-15]. For example, *Candida* cells that survive in prostheses after chemical or mechanical treatment form a biofilm in the presence of nutrients, causing a relapse of chronic infection [10, 11]. In addition, the oral cavity contains a wide variety of microbial species. Microbiota can interact intensely as the biofilm structure is formed to perform physiological functions and induce microbial pathogenesis [11, 16-18]. Biofilm-producing microorganisms are responsible for many persistent infections, and biofilm-associated diseases pose a significant problem in society, both economically and health-wise. However, an almost universal feature of biofilms is their resistance to chemical and physical injury, i.e., resistance to antibiotics and antifungals by various methods, including limited drug penetration to microorganisms, reduced growth rate, and expression of resistance genes. Therefore, it makes it very difficult to fight in clinical settings and is a burden that must be overcome regarding human health [5, 7, 9, 11, 19-21]. The role of bacterial biofilms in disease has been investigated in detail for several years, and there is substantial literature on their structure and properties. Since biofilm formation provides resistance to antimicrobial agents, the biofilm-forming capacity of *Candida* species, which are opportunistic pathogens, is critical for infection. Therefore, further recognition and understanding of *Candida* biofilms are crucial in studying human candidiasis. Studies on oral yeast load and yeast diversity in young individuals are limited in the literature. Özcan Ateş [22] has brought new information to the literature on the subject between 2018 and 2020. There is no information in the literature about the antifungal resistance and biofilm formation capacity of yeasts isolated from the oral mycobiota of young individuals. Studies in the literature have generally determined the antifungal resistance and biofilm-forming capacity of fungi isolated from individuals with various diseases. Therefore, this study aims to determine the antifungal resistance and biofilm formation capacity of yeast isolates isolated from the mouths of young individuals.

Materials and Methods

Sampling

The study obtained 133 yeast isolates identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) from 17 participants aged 18-25. Yeast isolates in stock culture were resuscitated at 37°C using Sabouraud Dextrose Broth (SDB) (NCM0147, Neogen, USA) medium. *Candida* genus isolates were cultivated on HiCrome™ Candida Differential Agar (M1297A, Himedia, India) medium, and *Wickerhamomyces subpelliculosus* (Kurtzman) Kurtzman, Robnett & Bas.-Powers and *Pichia manshurica* Saito (1914) isolates were cultivated on Sabouraud Dextrose Agar (SDA) medium (NCM0147, Neogen, USA) and their purity was checked.

Determination of Biofilm Forming Capacity by Congo Red Agar (CRA) Method

Congo Red Agar (CRA) method was performed as described in previous studies [7-9, 23]. Yeast isolates were first taken from the stock cultures, cultured in SDA medium, and incubated for 24 hours at 37°C. Then each culture was inoculated into CRA medium (Congo red 0.8 g/L (HiMedia, India), Brain Heart Infusion Broth 37 g/L (Oxoid, England), Agar 10 g/L (Liofilchem, Italy), Sucrose 50 g/L) and incubated at 37°C for 24–48 h. After incubation, black or purple-coloured colonies with a dry crystal consistency were interpreted as positive biofilm-producing strains and red-coloured colonies as negative for biofilm production. This analysis was carried out in triplicate.

Determination of Biofilm Forming Capacity by Tube Method (TM)

A qualitative assessment of biofilm formation was performed as described in previous studies [7, 9, 24]. Stock yeast cultures were resuscitated in 5 mL of SDB medium. 10 µL of resuscitated culture was cultivated SDB containing 8% glucose and incubated at 37°C for 48 hours. After incubation, the tubes were emptied, washed with Phosphate Buffered Saline (PBS) (pH-7.2), and dried. The tubes were stained with 0.1% v/v crystal purple (HiMedia, India). Tubes were washed with deionized water to remove excess dye. The tubes were then dried in the inverted position for biofilm determination. Biofilm formation was considered positive when visible film covered the wall and bottom of the tube. Ring formation at the liquid interface was not considered biofilm. The tube containing only sterile SDB was used as a negative control. This analysis was carried out in triplicate.

Determination of Biofilm Forming Capacity by Tissue Culture Plate (TCP) Method (Crystal Violet Method)

Quantitative biofilm formation was evaluated by modifying the methods described by Muadcheingka and Tantivitayakul [25] and Kıvanç and Er [26]. Yeast isolates were resuscitated overnight at 37°C in 5 mL of medium. Revived cultures were adjusted to OD600 = 1.0 (10^7 cells/mL) in an SDB medium containing 8% glucose. Then, 200 µL of inoculated SDB medium containing 8% glucose was added to

the wells of 96-well flat-bottom microplates. Microplates were incubated at 37°C for 48 hours. After incubation, the microplates were washed 3 times with sterile physiological saline (PS, 0.85% NaCl). After washing, 200 µL of 99% methanol (Merck, Germany) was added for fixation and incubated for 15 minutes. The plates were then emptied and dried at room temperature. Afterwards, 200 µL of 1% (v/v) crystal violet was added to each well and incubated for 15 minutes. After incubation, the microplates were washed twice with sterile distilled water, and the plates were dried at room temperature. Then, 200 µL of 33% acetic acid (Merck, Germany) was added to the plates and evaluated in a microplate reader (Thermo Multiscan FC) at 570 nm. Biofilm formation was evaluated with the following formula: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$), and strong biofilm production ($4.OD_{nc} < OD_s$) [27]. The study was carried out in two parallels and three repetitions.

Antifungal Disk Diffusion Method

In vitro antifungal resistance of 133 resuscitated isolates against fluconazole (25 mcg) (SD232-5CT, Himedia, India) antifungal disc according to NCCLS M44-A [28] standard method Mueller-Hinton Agar + 2% Glucose, 0.5 µg/mL Methylene Blue Agar (MHA+GMB) (M1825, Himedia, India) medium. To compare the antifungal resistance of the isolates, *C. albicans* (C.P. Robin) Berkhout ATCC 10231, *C. albicans* ATCC 14053, *C. albicans* ATCC 24433, *C. albicans* ATCC 90028, *Candida tropicalis* (Castell.) Berkhout ATCC 1021 *Candida parapsilosis* (Ashford) Langeron & Talice ATCC 22019, *Cryptococcus neoformans* var. *grubii* Franzot et al. ATCC 90112 were used as reference strains. The isolates were first resuscitated from stock cultures by seeding in an SDA medium and incubating overnight at 37°C. The inoculum of the resuscitated isolates was adjusted to 0.5 McFarland ($1\text{-}5x10^6$ cells/mL) with a McFarland densitometer with PS. Within 15 minutes after adjusting the turbidity of the inoculum suspension, the suspension was inoculated with a sterile cotton swab (swab) by rubbing the swab evenly over the entire agar surface of the dried surface of the MHA+ GMB petri plate. Fluconazole antifungal disc was placed on the agar surface 15 minutes after inoculation. The plates were then incubated at 37°C for 24 hours. Poorly grown ones were incubated for 48 hours. After incubation, zone diameters were measured with a caliper (KMP150, OEM, CHINA). The study was carried out in 2 parallels.

Determination of Minimum Inhibitory Concentration of Antifungal Agents by Gradient Test

Since 133 isolates were determined to be susceptible to fluconazole by the in vitro agar disc diffusion method, 20 isolates known as strong biofilm producers were selected to determine the Minimum Inhibitory Concentration (MIC). Fluconazole (0.016-256 mcg/mL, EM072, Himedia, India), itraconazole (0.002-32 mcg/mL, EM073, Himedia, India), clotrimazole (0.002-32 mcg/mL, EM144,

Himedia, India), nystatin (0.002-32 mcg/mL, EM145, Himedia, India) and amphotericin B (0.002-32 mcg/mL, EM071, Himedia, India) gradient test strips were performed to determine MIC values as specified in CLSI M27-A2 [29]. The inoculum suspension was adjusted as previously described. Within 15 minutes after adjusting the turbidity of the inoculum suspension, the suspension was inoculated with a sterile cotton swab (swab) by rubbing the swab evenly over the entire agar surface onto the dried surface of the petri plate containing RPMI 1640 agar containing 0.165 M MOPS + 2% glucose (M1972, Himedia, India). Fifteen minutes after inoculation, antifungal gradient test strips were placed on the 90 mm diameter agar surface as 1-2 pieces. The plates were then incubated at 37°C for 24 hours. Poorly grown ones were incubated for 48 hours. After incubation, MIC values were determined. Results were compared in line with NCCLS guidelines and previous studies.

Statistical Analysis

In evaluating the data, the Chi-square homogeneity test and the one-way and two-way analysis of variance were performed using SPSS Package Program (v23.0, IBM Corp) at a significance level of 0.05. Results are given as mean (M) ± standard deviation (sd).

Results

The biofilm-forming capacities of 133 yeast isolates isolated from the mouths of healthy young individuals were evaluated by three different methods: Congo Red Agar (CRA), Tube Method (TM) and Tissue Culture Plate (TCP) method. Comparisons of the methods used for biofilm formation determination of yeast isolates in three different methods are given in Table 1.

Table 1. *Comparison of the methods used to determine the biofilm-forming capacity of isolates at the species level*

Species	CRA	TM			TCP		
		Weak	Moderately strong	Strong	Weak	Moderately strong	Strong
C. albicans	2	29	2	0	42	10	4
C. dubliniensis	2	18	4	4	16	13	11
C. parapsilosis	0	2	6	19	2	0	27
C. inconspicua	0	2	0	0	0	1	2
P. manshurica	0	3	0	0	0	0	3
W. subpelliculosus	0	0	0	0	0	0	1
Total	**4**	**54**	**12**	**23**	**60**	**24**	**48**

As seen in Table 1, oral yeast isolates were composed of 43.60% *C. albicans*, 30.08% *C. dubliniensis*, 21.05% *C. parapsilosis*, 2.26% *C. inconspicua*, 2.26% *P. manshurica* and 0.75% *W. subpelliculosus.* It was determined that *C. dubliniensis* and *C. parapsilosis* were the most isolated species from 71 isolates of NCAC species, respectively. While it was determined that 47 isolates were strong and 24 isolates were moderately strong biofilm producers by the TCP method, 23 isolates were

determined to be strong biofilm producers, and 12 isolates were moderately strong biofilm producers by the TM method. In the CRA method, only four isolates were identified as biofilm producers. The comparison of the biofilm-forming capacities of 133 isolates at the species level with the TCP method is given in Figure 1.

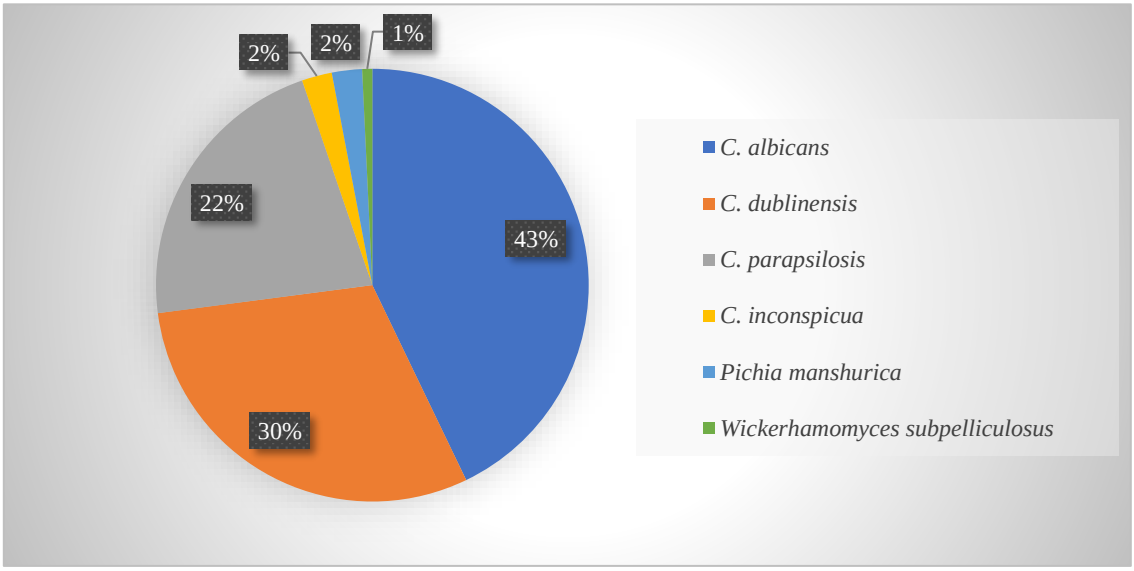

Figure 1. *Comparison of biofilm-forming species with the TCP method*

When the biofilm-forming capacities of the isolates were investigated, it was found that 99.25% of the isolates were biofilm producers with TCP, and 66.92% were biofilm producers with TM. 53.45% and 65% of *C. albicans* and *C. dubliniensis* isolates were biofilm producers by TM and 98.28% and 100% by TCP, respectively. For *C. parapsilosis*, 92.86% with TM and 100% with TCP were observed to be biofilm producers. The chi-square homogeneity test was used to find whether it varies according to the TM and TCP method used to determine the biofilm-forming capacity of the orally isolated species. As a result, the Pearson Chi-Square value was detected as $\chi 2=3.271$ and degrees of freedom (v=5). It was determined $P=0.658$ and found that it did not differ according to the TM and TCP method used in determining the species and biofilm-forming capacity ($P>0.05$). Regarding antifungal resistance, 133 isolates and seven standard strains were first evaluated against fluconazole antifungal by agar disc diffusion method. As a result, 133 yeast isolates were sensitive to fluconazole. Regarding antifungal resistance, 133 isolates and seven standard strains were first evaluated against fluconazole antifungal by agar disc diffusion method. As a result, it was determined that 133 yeast isolates were sensitive to fluconazole. The minimum and maximum values for fluconazole based on species are given in Table 2 in mm. Reference strains were also evaluated to assess the quality control range for antifungal resistance. Disk diffusion results of reference strains are shown in Table 3.

Table 2. *Maximum and minimum values in mm against fluconazole (FLC) antifungal*

Species (n)	**max**	**min**
C. albicans (58)	45.16	20.24
C. dubliniensis (40)	44.79	28.07
C. parapsilosis (28)	51.01	20.81
C. inconspicua (3)	38.36	27.87
P. manshurica (3)	36.42	32.93
W. subpelliculosus (1)	33.69	33.69

As seen in Table 2, *C. albicans* and *C. parapsilosis* isolates against fluconazole antifungal have a minimum inhibition zone of 20.24 and 20.81 mm and a maximum of 45.16 and 51.01 mm, respectively. While the minimum inhibition zone values of *C. dubliniensis* and *C. inconspicua* isolates against fluconazole are close to each other, their maximum values differ. On the other hand, there is no big difference between the minimum and maximum inhibition zones of *P. manshurica* isolates against fluconazole.

Table 3. *Quality control zone diameters (in mm) recommended by CLSI for fluconazole (FLC) antifungal and detected in our study*

Referans strains	**FLC (25 µg)**	**FLC (25 µg) (CLSI)**
C. albicans ATCC 10231	38.33±0.62	-
C. albicans ATCC 14053	39.47±0.57	-
C. albicans ATCC 24433	37.57±0.31	-
C. albicans ATCC 90028	37.97±0.57	28-39
C. parapsilosis ATCC 22019	32.25±0.52	22-33
C. tropicalis ATCC 1021	27.77±0.26	-
Cryptococcus neoformans ATCC 90112	37.79±0.01	-

Table 3 shows that the fluconazole antifungal used in the study was within the specified standards as a result of the evaluation with reference strains. Zone diameters regarding the participants and species are given in Figure 2. As a result of the statistical analysis made considering the participant and the species, it was determined that the inhibition zones against fluconazole (P=0.000) antifungal were statistically significant. As a result of determining that all isolates were susceptible to fluconazole by agar disc diffusion, 20 isolates, which are strong biofilm producers, were selected to determine their resistance to itraconazole, clotrimazole, amphotericin B and nystatin antifungals. The resistance of 20 isolates to itraconazole, clotrimazole, amphotericin B, and nystatin antifungals was evaluated with gradient test strips, and antifungal resistance was expressed as the MIC against each isolate. MIC values against 20 isolates and 2 quality control strains, which are strong biofilm producers, are given in Table 4.

ISSN: 2536-4383

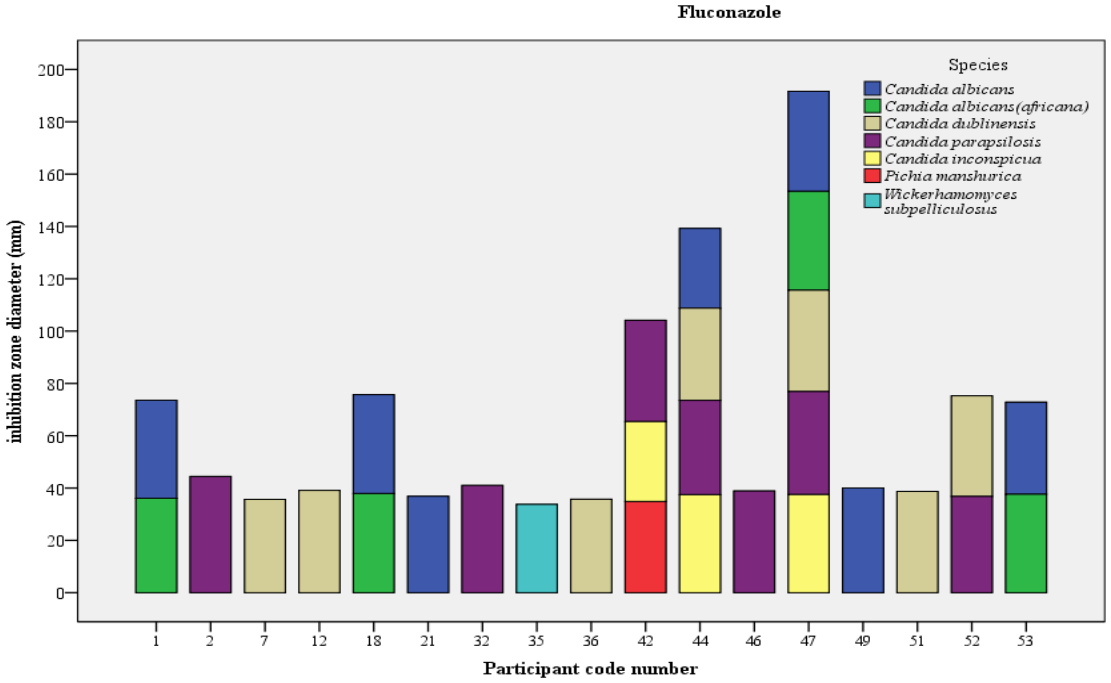

Figure 2. *Fluconazole zone diameters in mm against participant and yeast strain*

Table 4. *MIC values (in µg/ml).*

Isolates No	Species	Fluconazole	Itraconazole	Clotrimazole	Amphotericin B	Nystatin
S106	*C. albicans*	0.25 *	0.50*	0.38	4	>32
S111	*C. albicans*	0.25*	0.50*	0.25	4	>32
S131	*C. dubliniensis*	0.125	0.125	0.008	1	12**
S138	*C. dubliniensis*	0.50	0.38	0.023	8**	>32
S144	*P. manshurica*	8*	4*	0.125	2	8**
S145	*C. albicans*	0.75	0.25	0.023	8	>32
S148	*C. parapsilosis*	0.75	0.38	0.032	12	>32
S150	*C. parapsilosis*	1	0.38	0.032	8	>32
S154	*C. dubliniensis*	0.25	0.38	0.023	8**	>32
S161	*C. parapsilosis*	1.5	0.50	0.023	8**	>32
S164	*C. parapsilosis*	1.5	0.50	0.023	2	>32
S168	*C. parapsilosis*	0.75	0.50	0.016	12	>32
S207	*C. dubliniensis*	0.25	0.19	0.012	16	>32
S210	*C. dubliniensis*	0.25	0.25	0.012	16	>32
S216	*C. parapsilosis*	0.75	0.38	0.023	8	>32
S306	*C. parapsilosis*	0.25	0.125	0.012	16	>32
S308	*C. parapsilosis*	0.25	0.125	0.012	8	>32
S318	*C. parapsilosis*	0.125	0.19	0.016	8	>32
S342	*W. subpelliculosus*	1.5	0.19	0.023	12	>32
S470	*C. albicans*	1	0.25	0.38	4	>32
-	*C. albicans* ATCC 90028	0.38*	0.19*	0.125*	8	32
-	*C. parapsilosis* ATCC 22019	0.25*	0.19*	0.125*	2	6**

**detachable microcolonies, **small ellipse, resistant isolates*

Table 4 shows that 20 isolates that are strong biofilm producers and 2 quality control strains are susceptible to fluconazole antifungal.It was determined that 14 isolates were sensitive to itraconazole, and 3 were dose-dependent on clotrimazole antifungal. On the other hand, all tested isolates were found to be resistant to amphotericin B and nystatin antifungal. The obtained MIC values were interpreted according to NCCLS guidelines and previous studies [1, 4, 29-33], and the values are given in Table 5.

Table 5. *Interpretation of MIC values according to NCCLS guidelines and previous studies*

Antifungal agents	Interpretation criterion (µg/mL)		
	Susceptible	**Susceptible-dose dependent**	**Resistant**
Flukonazole	≤ 8 µg/mL	16–32 µg/mL	≥ 64 µg/mL
Itrakonazole	≤ 0.125 µg/mL	0.25-0.5 µg/mL	≥ 1 µg/mL
Klotrimazole	≤ 0.125 µg/mL*	0.25-0.5 µg/mL*	≥ 1 µg/mL*
Amphotericin B	≤ 1 µg/mL	-	> 1 µg/mL
Nystatin	-	-	≥ 16 µg/mL

* Evaluated by the manufacturer's recommendations.

When the standard strains and the interpretation criteria given in Table 5 are evaluated, the azole group antifungal results show the accuracy of the tests. However, the quality control results of amphotericin B and nystatin gradient tests are out of the manufacturer's results. Therefore, although 18 of 20 isolates were resistant to nystatin and 19 of 20 were resistant to amphotericin B, different methods must confirm these values.

Discussion

In this study, the biofilm-forming capacity of yeast isolates obtained from 17 participants aged 18-25 was examined by 3 different methods. It was determined that the most sensitive method among the applied methods for determining the biofilm formation potential was TCP, followed by TM and CRA methods. The results of this study are consistent with the results of the studies by Gogoi et al. [34] and Shrestha and Shakya [9]. Gogoi et al. [34] identified 115 bacterial isolates from different clinical samples, such as blood, and urine from urinary catheters, endotracheal tubes, tracheal aspirates, and drainage catheters by standard microbiological methods. The isolates they identified and non-biofilm-forming *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) and biofilm-forming *Pseudomonas aeruginosa* (ATCC 27853) cultures were evaluated by TCP, TM and CRA methods in determining the biofilm formation. They determined that TCP, TM and CRA methods were 61.7%, 41.7% and 18.2% in determining the biofilm-forming capacity of the isolates. In another study, Shrestha and Shakya [9] investigated the biofilm-forming capacities of 42 *C. albicans* isolates, which they isolated from 200 oral rinse samples collected with 10 mL of sterile PS for 1 minute, using three different methods. They defined the isolated *C. albicans* cultures according to their colony characteristics, simple staining, germ tube and chlamydospore characteristics. With the CRA method, they determined that 16 isolates were strong and moderate biofilm producers, while 26 isolates were

weak or not biofilm producers. They calculated that the sensitivity of the CRA method was 27.77%, the specificity was 16.66%, and the accuracy was 21.42%. They determined that 18 isolates were strong and medium biofilm producers, and 24 were not weak or biofilm producers in the TM method. They calculated that the sensitivity of the TM method was 71.4%, the specificity was 62.85%, and the accuracy rate was 64.28%. In the TCP method, they determined that 29 isolates were strong and moderate biofilm producers, and 13 isolates were weak or not biofilm producers. They stated that the TCP method is more sensitive, specific and accurate for quantitative biofilm screening than the TM and CRA methods. Comparing the results of this study with other studies, a high similarity is observed. In the study, 132 of 133 yeast isolates were found to be biofilm producers with TCP, while 89 isolates with TM were determined to be biofilm producers. As a result, it was determined that TCP is more sensitive in evaluating the biofilm-forming capacity of orally isolated yeasts. As a result of the study, it was determined that especially NCAC species isolated from the mouth are strong biofilm producers, while only 6.70% of *C. albicans* isolates are strong biofilm producers. The study results are similar to those of Pathak et al. [35] and Mohandas and Ballal [36]. Pathak et al. [35] evaluated biofilm formation on the surface of dental acrylic resin strips of single-species and multi-species combinations of *C. albicans* and NCAC. They isolated *Candida* isolates from many oral candidiases of neutropenic patients. After growing the isolates in an 8% glucose SDB medium, they were evaluated with crystal violet. As a result, they determined that single species (*C. glabrata* > *C. krusei* > *C. tropicalis* > *C. albicans*) and multi-species combinations (highest for *C. albicans* and *C. glabrata* and lowest for all four species combinations) had the biofilm-forming ability. The biofilm-forming ability of NCAC isolates was higher than *C. albicans* isolates isolated from multi-species oral candidiasis of neutropenic patients. Mohandas and Ballal [36] determined by the TM method that 81 (73%) of 111 *Candida* isolates out of 250 different clinical samples from patients treated in hospitals and nursing homes produced biofilms. They found that only 51% (25/49) of *C. albicans* isolates produced biofilm. They determined that NCAC species such as *C. krusei* and *C. tropicalis* formed stronger biofilms than *C. albicans*. Many protocols and media are in the literature for evaluating biofilm formation. Studies on media used to evaluate the biofilm formation of *Candida* species are available in the literature. Weerasekera et al. [37] evaluated the biofilm-forming capacity of *C. albicans* and *C. tropicalis* isolates using MTT and crystal violet (CV) methods in three different media (RPMI 1640, SDB, and yeast nitrogen base (YNB)). Researchers found that mono or dual growth was highest in SDB (20 g/L) medium with high sugar content, followed by YNB (18 g/L) and RPMI 1640 (2 g/L glucose) mediums. In addition, they determined that *C. tropicalis* species exhibited maximum adhesion in the YNB medium containing 100 mM glucose, while *C. albicans* and mixed *Candida* species achieved maximum adhesion with RPMI 1640 medium. Researchers stated that the medium is important in determining the biofilm-forming capacity of *Candida* species and that the methods should be standardized in this regard [37-39]. Mimicking the composition of human fluids rich in amino acids, RPMI 1640 medium is a rich medium containing many different

components, including high concentrations of L-Glutamine, L-Arginine, L-Asparagine, as well as vitamins and inorganic salts. [37, 40]. However, the glucose content of the RPMI 1640 medium is quite low compared to SDB and YNB mediums. For this reason, higher planktonic growth occurs in SDB medium with high glucose content compared to other mediums. On the other hand, SDB and YNB media are not rich in amino acids like RPMI 1640 media. However, the amino acid-rich composition of RPMI 1640 medium may promote a favourable biofilm formation. In addition, RPMI 1640 medium is recommended according to the NCCLS M27-A3 protocol for evaluating antifungal agents against planktonic cells [37]. Konecná et al. [41] investigated the effect of four different culture media on the biofilm biomass formation of *Candida* genus yeasts. Due to their clinical importance, they focused on *C. albicans* isolates in their studies. As a result, they stated that the presence of other components, such as amino acids or proteins, in the culture medium, along with glucose, helps to promote the transition of *Candida* yeasts to a stable form. They found that in vitro intact biofilm formation was increased, especially in *C. albicans* isolates, especially in the medium supplemented with fetal bovine serum (FBS). They stated that a culture medium with 10 g/L glucose and 10% (v/v) FBS added is important for the biofilm production of *C. albicans* isolates in vitro. Considering the Konecná et al. [41] study, this study used SDB containing 8% glucose. Especially *C. parapsilosis* strains were determined to be strong biofilm producers in vitro. For this reason, analyses were performed using an SDB medium containing 8% glucose in the study. The agar disc diffusion method evaluated the antifungal resistance of 133 yeast isolates according to the NCCLS M44-A standard. Then, considering the agar disc diffusion results, the resistance of 20 yeast isolates to 5 antifungal agents was investigated according to the NCCLS M27-A standard by selecting isolates with strong biofilm-forming ability. Accordingly, 20 yeast isolates and two quality control strains isolated from the mouths of healthy young individuals and determined to be strong biofilm producers were found to be susceptible to fluconazole antifungal. It was determined that 14 isolates were sensitive to itraconazole, and three were dose-dependent on clotrimazole antifungal. However, it was determined that the results of the amphotericin B and nystatin gradient tests were outside the MIC values determined by the manufacturer's recommendations in control of the quality control strains. Therefore, these values need to be verified by different methods. Kuriyama et al. [4] obtained 618 *Candida* isolates from 559 patients admitted to Dental Hospitals in Cardiff, Glasgow, Belfast and London (Eastman) between 2000-2003. They evaluated the antifungal resistance of these isolates against amphotericin B, nystatin, fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole using NCCLS M27-A guide broth microdilution method. They determined that 521 of the isolates they obtained were *C. albicans*, and 0.3% were resistant to fluconazole. They determined that the remaining 97 isolates were NCAC (*C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, other *Candida sp.*) species. They found that both *C. albicans* and NCAC isolates were susceptible to ketoconazole, miconazole and voriconazole antifungals. They determined that 23.7% of *C. glabrata* isolates were resistant to itraconazole. They reported little difference in the antifungal susceptibility of

Candida species isolated from patients who used and did not use antifungals before. Dhanasekaran et al. [42] evaluated 30 *Candida* isolates they isolated from dental plaque in terms of antifungal resistance against six clinically important antifungal agents such as amphotericin b, nystatin, clotrimazole, fluconazole, ketoconazole, and itraconazole by agar disc diffusion method. As a result, they determined that biofilm-forming isolates were significantly resistant to antifungal drugs compared to non-biofilm-forming *Candida* isolates. In our study, the antifungal susceptibility of only biofilm producer isolates was evaluated, and it was determined that they were susceptible to the azole group. The broth microdilution reference method, used to determine the MIC values of antimicrobial agents and adapted by CLSI, is a complex and time-consuming method to apply in the laboratory. Therefore, the gradient test method used to determine MIC values is the simple disk diffusion method. The results of this method have been used recently because they are compatible with liquid microdilution [1]. Koga-Ito et al. [43] evaluated the resistance to amphotericin B, 5-flucytosine, fluconazole, and itraconazole antifungals of 30 *C. albicans* isolated from denture-associated oral candidosis patients and 30 *C. albicans* isolates from control individuals using the CLSI reference method and gradient test method. The researchers found that the agreement between the two methods was 66.67% for amphotericin B, 65% for flucytosine, 53.33% for fluconazole, and 45% for itraconazole. The researchers stated that the gradient test method could be an alternative to the routinely used susceptibility test due to its simplicity and similarity of sensitivity. Song et al. [1] determined the antifungal resistance of 39 *C. albicans*, 5 *C. glabrata* and 3 *C. tropicalis* isolates they isolated from 45 patients with oral candidiasis and the *C. albicans* ATCC 90028 reference strain using the gradient test method. The MIC values against fluconazole, itraconazole, voriconazole and amphotericin B antifungals were determined by gradient test after 24 hours of incubation of each isolate on RPMI 1640 agar. They found that all *Candida* isolates were susceptible to amphotericin B and voriconazole. However, they determined that all five *C. glabrata* isolates were resistant to itraconazole, and two were resistant to fluconazole. In conclusion, the gradient test is a simple and effective method for antifungal susceptibility testing of *Candida* species isolated from patients with oral candidiasis. The results stated that amphotericin B and voriconazole are effective alternatives in treating oral candidiasis. Therefore, in our study, the sensitivity of biofilm producer yeasts isolated from the mouths of healthy individuals to antifungals was determined by a gradient test. CLSI has determined a cut-off value (ECV) for some *Candida* (*C. albicans, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*) species by evaluating the results of epidemiological studies. Accordingly, these *Candida* species were determined as S≤2 and R>4 mg/ml for fluconazole and S≤1 and R>1 mg/mL for amphotericin. In itraconazole antifungal, S≤0.06 and R>0.06 mg/mL for *C. albicans, C. dubliniensis* and S≤0.125 and R>0.125 mg/mL for *C. parapsilosis* and *C. tropicalis* [44]. In line with these values, it was determined that 20 isolates were resistant to itraconazole and amphotericin B antifungals and sensitive to fluconazole antifungals. However, MIC values of amphotericin B antifungal need to be confirmed.

Conclusion

In summary, it was determined that the sensitivity of the TCP method, one of the three methods used to evaluate biofilm formation, is high. It was determined that 99.25% of the isolates formed biofilms with TCP. MICs of antifungals for oral yeast strains could be determined quickly and successfully against azole antifungals, except for amphotericin B and nystatin antifungals, using a simple gradient test method. In conclusion, biofilm formation as a virulence factor is important for human health, and more research is needed on opportunistic pathogenic fungi species found in both healthy and diseased individuals. In addition, further studies with more clinical isolates are required to investigate the antifungal resistance tendency in sick and healthy individuals. Finally, it is necessary to investigate the resistance of yeasts isolated from the mouths of diseased and healthy individuals to antifungals after forming a biofilm.

Acknowledgments -

Funding/Financial Disclosure This study was supported by Çanakkale Onsekiz Mart University Scientific Research Projects Coordination Unit with project number: THD-2020-3352.

Ethics Committee Approval and Permissions For this study, we received permission from the school administration and ethics committee approval from Çanakkale Onsekiz Mart University Clinical Research Ethics Committee (No: 18920478–050.01.04–E180031135).

Conflict of Interests The authors declared no conflict of interest.

Authors Contribution Gülçin ÖZCAN ATEŞ's contribution to the article is 70%, she planned the project, analyzed and wrote the article. Müşerref OTKUN's contribution to the article was 30%, she planned the project and wrote the article.

References

[1] Song, Y. B., Suh, M. K., Ha, G. Y., & Kim, H. (2015). Antifungal susceptibility testing with etest for *Candida* species isolated from patients with oral candidiasis. *Annals of Dermatology*, *27*(6), 715. https://doi.org/10.5021/ad.2015.27.6.715

[2] Niimi, M., Firth, N. A., & Cannon, R. D. (2010). Antifungal drug resistance of oral fungi. *Odontology*, *98*(1), 15-25. https://doi.org/10.1007/s10266-009-0118-3

[3] Whaley, S. G., Berkow, E. L., Rybak, J. M., Nishimoto, A. T., Barker, K. S., & Rogers, P. D. (2017). Azole antifungal resistance in *Candida albicans* and emerging non-albicans *Candida* species. *Frontiers in Microbiology*, *7*, 2173. https://doi.org/10.3389/fmicb.2016.02173

[4] Kuriyama, T., Williams, D. W., Bagg, J., Coulter, W. A., Ready, D., & Lewis, M. A. O. (2005). In vitro susceptibility of oral *Candida* to seven antifungal agents. *Oral Microbiology and Immunology*, *20*(6), 349-353. https://doi.org/10.1111/j.1399-302X.2005.00236.x

[5] Jiao, Y., Tay, F. R., Niu, L. N., & Chen, J. H. (2019). Advancing antimicrobial strategies for managing oral biofilm infections. *International Journal of Oral Science*, *11*(3), 1-11. https://doi.org/10.1038/s41368-019-0062-1

[6] Dhale, R. P., Ghorpade, M. V., & Dharmadhikari, C. A. (2014). Comparison of various methods used to detect biofilm production of *Candida* species. *Journal of Clinical and Diagnostic Research: JCDR*, *8*(11), DC18. https://doi.org/10.7860/JCDR/2014/10445.5147

[7] Khatri, S., Sumana, M. N., Mahale, R. P., & Kishore, A. (2015). Analysing three different screening methods for biofilm formation in clinical isolates of *Candida*. *Journal of Evolution of Medical and Dental Sciences*, *4*(83), 14515-14524. https://doi.org/10.14260/jemds/2015/2065

[8] Sida, H., Shah, P., Pethani, J., Patel, L., & Shah, H. (2016). Study of biofilm formation as a virulence marker in *Candida* species isolated from various clinical specimens. *International Journal of Medical Science and Public Health*, *5*(5), 842-6. http://journalsarchive.com/FILES/IJMSPH/04.%20Study%20of%20biofilm%20formation.pdf

[9] Shrestha, B. K., & Shakya, J. (2020). In-vitro evaluation of biofilm and hemolysis activity of *candida albicans* isolated from oral cavity. *International Journal of Applied Sciences and Biotechnology*, *8*(4), 394-399. https://doi.org/10.3126/ijasbt.v8i4.32971

[10] Lewis, K. (2012). Persister cells: molecular mechanisms related to antibiotic tolerance. Anthony R.M. Coates (Ed), *Antibiotic Resistance*, (pp. 121-133). Springer

[11] Martins, C. H. G., Pires, R. H., Cunha, A. O., Pereira, C. A. M., de Lacorte Singulani, J., Abrão, F., Moraes, T. D., & Mendes-Giannini, M. J. S. (2016). *Candida/Candida* biofilms. First description of dual-species *Candida albicans/C. rugosa* biofilm. *Fungal biology*, *120*(4), 530-537. https://doi.org/10.1016/j.funbio.2016.01.013

[12] Seneviratne, C. J., Wang, Y., Jin, L., Abiko, Y., & Samaranayake, L. P. (2010). Proteomics of drug resistance in *Candida glabrata* biofilms. *Proteomics*, *10*(7), 1444-1454. https://doi.org/10.1002/pmic.200900611

[13] Tumbarello, M., Posteraro, B., Trecarichi, E. M., Fiori, B., Rossi, M., Porta, R., Donati, K. D. G., Sorda, M. L., Spanu, T., Fadda, G., Cauda, R., & Sanguinetti, M., (2007). Biofilm production by *Candida* species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. *Journal of Clinical Microbiology*, *45*(6), 1843-1850. https://doi.org/10.1128/jcm.00131-07

[14] Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D. W., & Azeredo, J. (2011). Adherence and biofilm formation of non-*Candida albicans Candida* species. *Trends in Microbiology*, *19*(5), 241-247. https://doi.org/10.1016/j.tim.2011.02.003

[15] Tumbarello, M., Fiori, B., Trecarichi, E. M., Posteraro, P., Losito, A. R., De Luca, A., Sanguinetti, M., Fadda, G., Cauda, R., & Posteraro, B. (2012). Risk factors and outcomes of candidemia caused by biofilm-forming isolates in a tertiary care hospital. *PloS One*, *7*(3), e33705. https://doi.org/10.1371/journal.pone.0033705

[16] Kuramitsu, H. K., He, X., Lux, R., Anderson, M. H., & Shi, W. (2007). Interspecies interactions within oral microbial communities. *Microbiology and Molecular Biology Reviews*, *71*(4), 653-670. https://doi.org/10.1128/MMBR.00024-07

[17] Thein, Z. M., Seneviratne, C. J., Samaranayake, Y. H., & Samaranayake, L. P. (2009). Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses*, *52*(6), 467-475. https://doi.org/10.1111/j.1439-0507.2009.01719.x

[18] Kolenbrander, P. E., Palmer, R. J., Periasamy, S., & Jakubovics, N. S. (2010). Oral multispecies biofilm development and the key role of cell–cell distance. *Nature Reviews Microbiology*, *8*(7), 471-480. https://doi.org/10.1038/nrmicro2381

[19] Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9), 623-633. https://doi.org/10.1038/nrmicro2415

[20] Gulati, M., & Nobile, C. J. (2016). *Candida albicans* biofilms: development, regulation, and molecular mechanisms. *Microbes and İnfection*, *18*(5), 310-321. https://doi.org/10.1016/j.micinf.2016.01.00

[21] Miquel, S., Lagrafeuille, R., Souweine, B., & Forestier, C. (2016). Anti-biofilm activity as a health issue. *Frontiers in Microbiology*, *7*, 592. https://doi.org/10.3389/fmicb.2016.00592

[22] Özcan Ateş, G. (2020). *Ağızda Maya Yükünün Belirlenmesi ve İzole Edilen Candida Cinsi Mayaların Tanımlanması*. (Tez no: 621102) [Yüksek Lisans Tezi, Çanakkale Onsekiz Mart Üniversitesi].

[23] Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase-negative staphylococci. *Journal of clinical pathology*, *42*(8), 872-874. http://dx.doi.org/10.1136/jcp.42.8.872

[24] Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology*, *22*(6), 996-1006. https://doi.org/10.1128/jcm.22.6.996-1006.1985

[25] Muadcheingka, T., & Tantivitayakul, P. (2015). Distribution of *Candida albicans* and non-albicans *Candida* species in oral candidiasis patients: Correlation between cell surface hydrophobicity and biofilm forming activities. *Archives of Oral Biology*, *60*(6), 894-901. https://doi.org/10.1016/j.archoralbio.2015.03.002

[26] Kıvanç, M., & Er, S. (2020). Biofilm formation of *Candida* spp. isolated from the vagina and antibiofilm activities of lactic acid bacteria on the these *Candida* Isolates. *African Health Sciences*, *20*(2), 641-648. https://doi.org/10.4314/ahs.v20i2.12

[27] Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis, *115*(8), 891-899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

[28] National Committee for Clinical Laboratory Standards. (2004). *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline*. (NCCLS Standard No: M44-A). https://clsi.org/standards/products/microbiology/documents/m44/

[29] National Committee for Clinical Laboratory Standards. (2002). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*. (NCCLS Standard No. M27-A2). https://clsi.org/standards/products/microbiology/documents/m44/

[30] Espinel-Ingroff, A., & Pfaller, M. A. (1995). Antifungal agents and susceptibility testing. Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, DC, 1405-1414.

[31] Davey, K. G., Holmes, A. D., Johnson, E. M., Szekely, A., & Warnock, D. W. (1998). Comparative evaluation of FUNGITEST and broth microdilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans*. *Journal of Clinical Microbiology*, *36*(4), 926-930. https://doi.org/10.1128/JCM.36.4.926-930.1998

[32] Kronvall, G., & Karlsson, I. (2001). Fluconazole and Voriconazole Multidisk Testing of *Candida* Species for Disk Test Calibration and MIC Estimation. *Journal of Clinical Microbiology*, *39*(4), 1422-1428. https://doi.org/10.1128/jcm.39.4.1422-1428.2001

[33] Blignaut, E., Messer, S., Hollis, R. J., & Pfaller, M. A. (2002). Antifungal susceptibility of South African oral yeast isolates from HIV/AIDS patients and healthy individuals. *Diagnostic Microbiology and Infectious Disease*, *44*(2), 169-174. https://doi.org/10.1016/S0732-8893(02)00440-6

[34] Gogoi, M., Sharma, A., & Hazarika, N. (2015). Biofilm formation by bacterial isolates from patients on indwelling medical devices. *Indian Journal of Medical Microbiology*, *33*(2), 319. https://doi.org/10.4103/0255-0857.154896

[35] Pathak, A. K., Sharma, S., & Shrivastva, P. (2012). Multi-species biofilm of *Candida albicans* and non-Candida albicans *Candida* species on acrylic substrate. *Journal of Applied Oral Science*, *20*(1), 70-75. https://doi.org/10.1590/S1678-77572012000100013

[36] Mohandas, V., & Ballal, M. (2011). Distribution of *Candida* species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. *Journal of Global Infectious Diseases*, *3*(1), 4. https://doi.org/10.4103/0974-777X.77288

[37] Weerasekera, M. M., Wijesinghe, G. K., Jayarathna, T. A., Gunasekara, C. P., Fernando, N., Kottegoda, N., & Samaranayake, L. P. (2016). Culture media profoundly affect *Candida albicans* and *Candida tropicalis* growth, adhesion and biofilm development. *Memorias do Instituto Oswaldo Cruz*, *111*(11), 697-702. https://doi.org/10.1590/0074-02760160294

[38] McCourtie, J., MacFarlane, T. W., & Samaranayake, L. P. (1985). Effect of chlorhexidine gluconate on the adherence of *Candida* species to denture acrylic. *Journal of Medical Microbiology*, *20*(1), 97-104. https://doi.org/10.1099/00222615-20-1-97

[39] Samaranayake, L. P., MacFarlane, T. W., Lamey, P. J., & Ferguson, M. M. (1986). A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. *Journal of Oral Pathology & Medicine*, *15*(7), 386-388. https://doi.org/10.1111/j.1600-0714.1986.tb00646.x

[40] Kucharíková, S., Tournu, H., Lagrou, K., Van Dijck, P., & Bujdakova, H. (2011). Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. *Journal of Medical Microbiology*, *60*(9), 1261-1269. https://doi.org/10.1099/jmm.0.032037-0

[41] Konečná, K., Němečková, I., Diepoltová, A., Vejsová, M., & Janďourek, O. (2021). The Impact of Cultivation Media on the In Vitro Biofilm Biomass Production of *Candida* spp. *Current Microbiology*, 1-8. https://doi.org/10.1007/s00284-021-02452-6

[42] Dhanasekaran, D., Vinothini, K., Latha, S., Thajuddin, N., & Panneerselvam, A. (2014). Human dental biofilm: Screening, characterization, in vitro biofilm formation and antifungal resistance of

Candida spp. *The Saudi Journal for Dental Research*, *5*(1), 55-70. https://doi.org/10.1016/j.ksujds.2013.10.001

[43] Koga-Ito, C. Y., Lyon, J. P., & Resende, M. A. D. (2008). Comparison between E-test and CLSI broth microdilution method for antifungal susceptibility testing of *Candida albicans* oral isolates. *Revista do Instituto de Medicina Tropical de São Paulo*, *50*(1), 7-10. https://doi.org/10.1590/S0036-46652008000100002

[44] Arendrup, M. C., Friberg, N., Mares, M., Kahlmeter, G., Meletiadis, J., Guinea, J., Kahlmeter, G., Andersen, C.T., Arikan-Akdagli, S., Barchiesi, F., Chryssanthou, E., Hamal, P., Järv, H., Klimko, N., Kurzai, O., Lagrou, K., Lass-Flörl, C., Matos, T., Muehlethaler, K., Rogers, T. R., Velegraki, A., & Arikan, S. (2020). How to interpret MICs of antifungal compounds according to the revised clinical breakpoints v. 10.0 European committee on antimicrobial susceptibility testing (EUCAST). *Clinical Microbiology And Infection***,** *26*(11), 1464-1472. https://doi.org/10.1016/j.cmi.2020.06.007