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BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

Saprochaete/Magnusiomyces: identification, virulence factors, and antifungal susceptibility of a challenging rare yeast

Beyzanur Kaşaltı[1] · **Dolunay Gülmez**[1]

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Abstract

Saprochaete/Magnusiomyces is among rare yeasts which might emerge as causes of breakthrough infections and nosocomial outbreaks. Identification to the species level might be a challenge in clinical laboratories. Data on virulence factors are scarce and antifungal susceptibility testing methodology is not definite. The aim of this study was to confirm species identification of clinical *Saprochaete/Magnusiomyces* isolates, find out their virulence factors, and obtain antifungal minimum inhibitory concentrations with two reference methods. Of the 57 isolates included, 54 were *Saprochaete capitata* and four were *Saprochaete clavata* as identified by ID32C, MALDI-TOF MS, and sequencing. When tested using phenotypic methods, all isolates were negative for coagulase, hemolysis, acid proteinase, and phospholipase, 56.1% were positive for esterase, and 19.3% had intermediate surface hydrophobicity. All isolates formed biofilms, with 40.4% of the isolates producing more biomass than biofilm-positive reference strain *Candida albicans* MYA-274. Antifungal susceptibility testing needed an adjusted spectrophotometric inoculum than recommended in reference methods for *Candida/Cryptococcus*. In conclusion, *Saprochaete/Magnusiomyces* species could be identified using methods available in the clinical laboratories. Despite the disadvantages of the phenotypic methods, esterase positivity was observed for the first time. A high biomass production was observed in biofilms. The need for standardization of antifungal susceptibility testing was brought to attention.

Keywords Rare yeast · *Saprochaete clavata* · *Magnusiomyces capitatus* · Esterase · Biofilm · Antifungal susceptibility testing

Introduction

Saprochaete/Magnusiomyces species are rare causes of human infection [1]. They may cause invasive and mortal infections, especially in hemato-oncology patients and other immunocompromised hosts; nevertheless, they may be encountered in immunocompetent people [1, 2]. There are sporadic cases and nosocomial outbreaks caused by *Saprochaete/Magnusiomyces* species in different countries of the world [1–4]. Bloodstream infections as well as infections involving organs such as liver, lung, endocardium, brain, bone, joint, and skin were reported [1, 5, 6]. Some of these cases emerged as breakthrough infections, predominantly under echinocandins [1, 7].

Taxonomically, *Saprochaete/Magnusiomyces* species are classified under the Ascomycetes clade, Saccharomycetes class, order Saccharomycetales, and Dipodascaceae family [8, 9]. Different genera names, such as *Geotrichum*, *Blastoschizomyces*, and *Saprochaete* for anamorphs and *Dipoascus* and *Magnusiomyces* for teleomorphs, were used in the literature [1, 9]. The current nomenclature accepted for the most common clinical isolates are *Magnusiomyces capitatus* (An: *Saprochaete capitata*) and *Saprochaete clavata* (Tl: *Magnusiomyces clavatus*) [1, 8]. In order to avoid confusion, *S. capitata* and *S. clavata* were preferred throughout the text.

S. capitata and *S. clavata* are difficult to identify at the species level as their macroscopic and microscopic morphology is similar [1, 10]. They form white or cream yeast-like colonies which are smooth to wrinkled, irregularly spreading colonies in which the aerial hyphae become evident as they mature. Microscopically, yeast cells, true hyphae, pseudohyphae, abundant annelloconidia, and

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✉ Dolunay Gülmez
dolunayglm@gmail.com; dolunay@hacettepe.edu.tr

[1] Medical Microbiology Department, Hacettepe University Faculty of Medicine, Ankara, Turkey

few arthroconidia can be seen on cornmeal Tween 80 medium [1, 8, 9]. *Saprochaete/Magnusiomyces* species are urease negative and have few different biochemical properties such as salicin and cellobiose positivity of *S. clavata* [1, 8]. Apart from these conventional tests, matrix-assisted laser desorption/ionization mass spectrophotometry (MALDITOF-MS) and gene sequencing methods can be used for species-level identification. Accuracy of both methods relies on the database used. MALDI-TOF MS successfully identified most fungi including *Saprochaete/Magnusiomyces* when larger and more reliable databases are used [1, 10]. Gene sequencing is accepted as the gold standard for microbial identification, and the ITS is accepted as the primary fungal barcode for fungal identification [11]. However, some studies could not differentiate *Saprochaete/Magnusiomyces* species using only ITS sequences, due to unreliable databases which contained misidentified isolates and/or insufficient length of the sequences used [10]. Analysis of additional gene sequences such as *Rpb2*, *Tef1α*, and *Act*, whole genome analysis, and phylogenetic trees were proposed to overcome these problems [1, 3, 8, 9]. As correctly differentiating the two species is the first step in determining the differences in epidemiology, prognosis, virulence, and management, understanding the reliability of the identification methods in clinical microbiology laboratories is fundamental.

Saprochaete/Magnusiomyces infections might have very high mortality rates, which are attributed to the vulnerability of the host and their antifungal nonsusceptibility [1, 6]. They are considered as low virulent yeasts which rarely cause disease in humans [1]. Still, as far as we know, virulence factors which they harbor are hardly investigated, except in limited studies that reported secreted acid proteinase activity [12] and biofilm production [13]. There is no data on the most common fungal virulence factors such as the production of coagulase, phospholipase, and esterase; hemolysis ability; and surface hydrophobicity. The presence and expression of variable virulence factors and their contribution to pathogenesis are yet to be explained.

Reference antifungal susceptibility testing methods were standardized for *Candida* and *Cryptococcus,* and details of testing when used for *Saprochaete/Magnusiomyces* are not definitive [14, 15]. Besides, neither clinical breakpoints nor epidemiological cutoff values have been determined for *Saprochaete/Magnusiomyces*. Still, echinocandin minimum inhibitory concentration (MIC) values are high and *Saprochaete/Magnusiomyces* isolates are considered resistant to echinocandins in vitro and in vivo. Except for fluconazole, which yields high MICs in *Saprochaete/Magnusiomyces*, azole MICs are variable in individual strains [1]. Reference methods are recommended to test the antifungal susceptibility of *Saprochaete/Magnusiomyces*, in order to obtain reproducible and comparable MIC values [1].

In this study, we aimed to confirm species identification, reveal virulence factors, determine antifungal susceptibility of clinical *Saprochaete/Magnusiomyces* isolates, and contribute to the knowledge of a rare fungal pathogen.

Materials and methods

Clinical isolates

Fungal isolates obtained from clinical specimens between 2006 and 2021 at Hacettepe University Faculty of Medicine Hospitals Microbiology Laboratory were included. The hospitals are a 1200-bed tertiary-care complex with medical, surgical, and oncological intensive care units and bone-marrow and solid-organ transplantation wards and are a center for patients with chronic pulmonary diseases, patients needing immune suppressive treatment such as malignancies, rheumatological diseases, and immune disorders. The isolates were identified by colony morphology, microscopic morphology on corn meal Tween 80 medium, negative urease test, and assimilation profile detected by ID32C (BioMérieux, France) at the Mycology Laboratory. Brain heart infusion broth with 20% glycerol was used to store isolates at −20 °C. Isolates which were identified as *Saprochaete*, *Blastoschizomyces*, or *Geotrichum* were subcultured on Sabouraud dextrose agar (SDA; OXOID, UK) and were checked for viability, purity, colony morphology, and morphology on Corn Meal Tween 80 medium. They were selected for further tests when the identification was compatible with *Saprochaete/Magnusiomyces*. If multiple isolates from a single patient were available, the first available isolate from a patient was included.

Species identification

The isolates were identified using MALDITOF-MS according to the manufacturer's recommendations (Bruker Corporation, USA). Briefly, a sample from a colony grown overnight on Sabouraud dextrose agar was placed on target, covered by 1 µl 70% formic acid, left to dry, and then covered by 1 µl matrix solution. Matrix solution was prepared by dissolving 2.5 mg α-cyano-4-hydroxycinnamic acid (HCCA, Sigma 70990, UK), 75 µl methanol, and 150 µl organic solvent (475 µl water, 500 µl acetonitrile, 25 µl trifluoroacetic acid). Target was examined using MALDIBiotyper (Bruker Corporation, USA), and scores were noted.

For sequencing, DNA was extracted using a "heat shock" method [16]. Polymerase chain reaction for ITS and *Rpb2* was performed as described elsewhere [8]. Sequences of ITS region from all isolates were analyzed for species

identification. In case the ITS sequence was not sufficient to differentiate the species, *Rpb2* gene sequence was evaluated additionally [8]. Products were purified (PureLink PCR Purification Kit, Thermo Fisher Scientific, USA) and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher Scientific, USA). The sequences were checked (Bioedit 7.2.5 Sequence Alignment Editor, Ibis Therapeutics, USA) and compared to National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and International Society for Human and Animal Mycology (ISHAM) databases (https://its.mycologylab.org/page/Alignment, ITS only) to determine species identification. In order to visualize species distribution, a phylogenetic tree was constructed using ITS sequences of the isolates using Molecular Evolutionary Genetics Analysis software (MEGA 11, USA) for the maximum likelihood (ML) method (Tamura et al. 2021). The corrected Akaike information criterion (AICc) was chosen to estimate the best nucleotide substitution model by jModelTest v2.1.6 (Posada 2008). Bootstrap support value was calculated using 1000 replicates for the tree.

Screening for virulence factors

Saprochaete/Magnusiomyces isolates were tested for virulence factors observed in fungal pathogens phenotypically using conventional methods. Tests were repeated three times for each isolate.

Coagulase activity was evaluated after incubation in rabbit and human plasma supplemented with EDTA after 2, 4, 6, and 24 h of incubation at 37 °C [17–19].

Hemolysis was evaluated on SDA supplemented with 7% sheep blood or horse blood. Plates were incubated at 37 °C in 5% CO_2 for 48 h [17].

Acid proteinase was investigated on solid media containing 1% bovine serum albumin [20, 21]. Briefly, 20 g glucose, 1 g KH_2PO_4, and 0.5 g $MgSO_4$ were dissolved in 800 ml water. The solution was autoclaved, cooled to 50 °C, then mixed with filter-sterilized 2 g bovine serum albumin prepared in 200 ml water, and poured into the Petri plates. A 0.5 McFarland suspension was prepared from each isolate, and 10 µl of the suspension was placed on the medium. The plates were incubated at 37 °C for 6 days. The presence of a clear lysis zone around the growing microorganism was evaluated as positive.

Phospholipase activity was tested on media containing 8% egg yolk and citric acid disodium phosphate buffer [20, 21]. A mixture of 13 g SDA,11.7 g NaCl, and 0.111 g $CaCl_2$ was autoclaved and cooled to 60 °C, sterile solutions of 8 ml 0.1 M citric acid ($C_6H_8O_7H_2O$), and 8 ml 0.2 M Na_2HPO_4 and 20 ml homogenized egg yolk were added. pH was adjusted to 4.2, and the final solution was transferred to sterile plates. Ten µl from a fungal suspension of 0.5 McFarland density was inoculated, and the plates were evaluated after incubation at 37 °C for 4 days. The presence of precipitation zones was sought for positive activity.

Esterase activity was observed on Tween 80 agar. A mixture of 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$, and 1 L of distilled water was autoclaved. Separately autoclaved 5 ml Tween 80 was added to this mixture at 50 °C and adjusted to pH = 6.8. After inoculation, plates were incubated at 30 °C for 10 days [21, 22]. Precipitation around the inoculum was a positive result.

Surface hydrophobicity was tested by hydrocarbon adhesion method [21]. A solution of each isolate was prepared in PUM (phosphate, urea, $MgSO_4$) buffer and adjusted to 1×10^7 cfu/ml, and 2 ml of the solution was transferred to 2 tubes. The first was used as a control, and 0.5 ml n-hexadecane was added to the latter. The tubes were incubated in a 37 °C water bath for 10 min, were vortexed, and were incubated for a further 30 min. A sample from the lower aqueous phase was obtained via a pipette, absorbance was measured at 660 nm, and OD values of two tubes were compared.

Biofilm formation of the isolates was investigated. Biofilm biomass was detected using crystal violet (CV), and metabolic activity was explored using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide (XTT) [23, 24]. Biofilm-forming ability of each isolate was interpreted as negative or positive (weak, intermediate, or strong) according to OD values compared to negative controls [25]. In addition, OD values of the isolates were compared to and were given as percentage of the activity of biofilm positive reference strain (*Candida albicans* MYA-274) which was accepted as 100%.

In vitro antifungal susceptibility testing

Minimum inhibitory concentration (MIC) values of *Saprochaete/Magnusiomyces* isolates against amphotericin B, fluconazole, voriconazole, and micafungin were determined using the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference microdilution methods [14, 15]. For each isolate, inoculum was prepared in 5 ml saline containing 200 µl Tween 20 (Biomatik, Canada). Cell counts were performed under the microscope for the appropriate concentration, and the percent transmission of light for the suspension was measured at 530 nm. MIC values were noted after 24 and 48 h of incubation. Quality control strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used in each run as recommended.

Results

Identification of isolates

A total of 57 isolates were included in the study. Of these, 53 were identified as *S. capitata*, and four were identified as *S. clavata* using conventional methods and ID 32C.

Identification scores obtained by MALDITOF-MS were ≥ 2 for 54 (*S. capitata n* = 52, *S. clavata n* = 2) and between 2 and 1.7 for two (*S. capitata n* = 1, *S. clavata n* = 1) isolates. Only one *S. clavata* isolate yielded scores below 1.70, with no other genera/species identification options given by the system.

ITS sequences evaluated in BLAST and ISHAM databases confirmed the genus and species identification for all *Saprochaete* isolates. For the four *S. clavata* isolates, ITS gene sequences also had high scores for *S. capitata*, especially in the BLAST database ("percent identity" scores (> 99%). Therefore, *Rpb2* sequences were also investigated for reliable differentiation of *S. clavata* which confirmed the identification. Moreover, the phylogenetic tree of ITS sequences showed these four *S. clavata* isolates clustered separately from the other isolates, which were identified as *S. capitata* (Supplementary Figure 1).

Virulence factors

Coagulase, hemolysis, acid proteinase, and phospholipase were negative for all *Saprochaete* isolates using phenotypic tests under given conditions.

Esterase activity was positive for 32 (56.1%) isolates (Supplementary Figure 2). Of these, 27 were *S. capitata*, and three were *S. clavata*.

Surface hydrophobicity was intermediate for 11 (19.3%) and weak for 46 (80.7%) isolates. All 11 isolates with intermediate surface hydrophobicity were *S. capitata*.

To evaluate biofilm-forming ability, CV was used to detect biomass formation, and XTT was used to understand metabolic activity. All isolates showed strong biofilm-forming ability with both methods when OD values were compared to negative controls. In order to accomplish a more detailed classification, OD values of the isolates were compared with the OD value of the biofilm-positive reference strain, *C. albicans* MYA-274, accepting the CV or XTT OD value of the latter as 100%. Metabolic activity was mostly lower than the reference strain, as the majority of the biofilms (*n* = 41, 71.9%) had a metabolic activity at 26–50%. However, biomass produced by 23 (40.4%) *Saprochaete/Magnusiomyces* isolates was higher than *C. albicans* MYA-274 (Table 1).

Antifungal susceptibility testing

Fungal suspensions consisting of 2–5 × 10^6 cells/ml were prepared using cell counts under the microscope, which revealed variable morphologies for *Saprochaete/Magnusiomyces* cells (Supplementary Figure 3). The percent transmittance of light for the solutions containing appropriate numbers of cells ranged between 62.0 and 65.8%, which was lower than *Candida* solutions of 0.5 McFarland (expected transmittance 80-82%) [26].

For *Saprochaete/Magnusiomyces* isolates included in the study, MIC values of amphotericin B, fluconazole, voriconazole, and micafungin obtained using both CLSI and EUCAST methods were noted after 24 and 48 h of incubation. For the CLSI method, 24 h incubation was insufficient, and 48 h results were used. The EUCAST method recommends an OD value of ≥ 0.200 in the growth control well for sufficient growth [15]. Only 3 of the 57 isolates reached sufficient growth after 24 h incubation. Except for these three, 48 h results were taken into account for the 54 isolates. MIC results are summarized in Table 2.

MIC values obtained by the EUCAST method were within 2 double-dilutions lower in 5 isolates (8.8%) for amphotericin B and in 1 isolate (1.8%) for micafungin, compared with those obtained by the CLSI method. For fluconazole, voriconazole, and micafungin, 56.1%, 84.2%, and 66.7% of the isolates had ≥ 2 double-dilution higher MIC values with EUCAST compared to CLSI MICs, respectively. For the rest of the isolates, the MICs obtained by

Table 1 Metabolic activity (XTT) and biomass production (CV) in biofilms of *Saprochaete* isolates compared to biofilm-positive *C. albicans* MYA-274 (100%).

Method	Microorganism	≤ 25%		26–50%		51–75%		76–100%		> 100%		Total (*n*)
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	
XTT	*S. capitata*	3	5.7	40	75.5	6	11.3	4	7.5	–	–	53
	S. clavata	–	–	1	25.0	–	–	2	50.0	1	25.0	4
	Total	3	5.3	41	71.9	6	10.5	6	10.5	1	1.8	57
CV	*S. capitata*	–	–	10	18.9	6	11.3	14	26.4	23	43.4	53
	S. clavata	–	–	1	25.0	3	75	–	–	–	–	4
	Total	–	–	11	19.3	9	15.8	14	24.6	23	40.4	57

XTT 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide, *CV* crystal violet

Table 2 Minimum inhibitory concentration (MIC) values (µg/ml) of *Saprochaete/Magnusiomyces* isolates included the study using two reference methods: Geometric mean (GM), MIC50, MIC90 and MIC range

Microorganism		CLSI				EUCAST			
		AMB	FLU	VOR	MCF	AMB	FLU	VOR	MCF
S. capitata ($n = 53$)	GM	1.74	3.33	0.11	3.65	0.95	8.21	0.93	18.72
	MIC_{50}	2	4	0.125	4	1	8	1	32
	MIC_{90}	2	8	0.25	16	1	16	4	32
	Range	1-2	0.25-16	0.016-0.5	0.5 - > 16	0.5-2	0.5-64	0.03-4	2 - > 16
S. clavata ($n = 4$)	GM	1.41	2.83	0.07	2.00	0.84	16.00	1.00	5.66
	MIC_{50}	–	–	–	–	–	–	–	–
	MIC_{90}	–	–	–	–	–	–	–	–
	Range	1-2	1-16	0.016-1	1-4	0.5-1	4-64	0.25 - > 8	0.5 - > 16
Total ($n = 57$)	GM	1.71	3.06	0.11	3.50	0.94	8.61	0.94	17.21
	MIC_{50}	2	4	0.125	4	1	16	1	> 16
	MIC_{90}	2	8	0.25	16	1	32	4	> 16
	Range	1-2	0.25-16	0.016-1	0.5 - > 16	0.5-2	0.5-64	0.03 - > 8	0.5 - > 16

AMB amphotericin B, *FLU* fluconazole, *VOR* voriconazole, *MCF* micafungin

the EUCAST and CLSI methods were distributed in the ±1 double-dilution range.

Discussion

Identification of *Saprochaete* isolates to genus level using macroscopic and microscopic morphology is applicable. However, species-level identification needs more tests, which might be a challenge for clinical laboratories. Isolates included in our study could successfully be identified to species level using a commercial kit based on biochemical reactions including carbohydrate assimilation tests (ID32C, BioMerieux, France) and MALDI-TOF MS, both of which might be available in routine clinical microbiology laboratories. Different commercial systems based on biochemical reactions are available in clinical laboratories for fungal identification. ID32C was successfully used to identify *S. capitata* [7] and failed to identify *S. clavata* [27] in previous studies, which used sequencing for confirmation of the identification. Nevertheless, all isolates included in our study were successfully identified at the species level, including four *S. clavata* isolates. Other commercial biochemical tests such as VITEK2 (BioMerieux, France), API20C (BioMerieux, France), and AuxaColor (BioRad, France) were also used to identify *Saprochaete/Magnusiomyces* with variable performances [28].

MALDI-TOF MS has been a promising method for microbiological identification and, with the expansion of the databases, might differentiate fungi to species level successfully, including *Saprochaete/Magnusiomyces* [3, 6, 7, 29]. The MALDI-TOF MS system used in this study (Bruker Diagnostics, USA) recommends a score of ≥ 2.0 for reliable species identification, states that a score of < 2.0–1.7 is reliable for genus identification, and considers scores < 1.7 as unreliable. Although MALDI-TOF MS generated a low score (< 1.7) for one of the *S. clavata* isolates, the database gave no other identification options. Kolecka et al. [29] also reported a *S. clavata* isolate with a MALDI-TOF MS identification score of < 1.7, for which the identification was confirmed with ITS sequencing. Improving the database using spectra from strains of confirmed identification might still be needed to eliminate doubt for *Saprochaete/Magnusiomyces* species.

In this study, ITS sequences were used to confirm the identification of *Saprochaete/Magnusiomyces* isolates, using two different databases. *S. capitata* isolates were identified without hesitation, as sequences yielded high scores in both databases. However, ITS sequences of four *S. clavata* isolates had low scores and/or high scores of both *S. clavata* and *S. capitata*. ITS sequences of these two species show high similarity (96%) which might cause misidentifications. Additional analysis of *Rpb2* sequence, which was found to be the best marker for differentiation among other candidates such as LSU, *Tef1α*, and *Act* previously, was used in our study [8]. All four isolates were identified as *S. clavata* when *Rpb2* sequences were compared in the BLAST database. In order to support the identification, a phylogenetic tree was built using ITS sequences, and the four *S. clavata* isolates clustered separately.

Fungal taxonomy and nomenclature are very dynamic research areas with massive current changes. Different genus and species names were proposed and used for *Saprochaete/Magnusiomyces* in the past decades [1, 8, 9]. Accurate identification of clinically important species in the routine microbiology laboratories is critical to reveal the real extent of the problem caused by infectious agents. *S. capitata* and *S. clavata* were described as the most common clinical isolates causing human infection, and we did not detect any other species in our culture collection.

Saprochaete/Magnusiomyces might cause infections with high mortality, even in immunocompetent hosts [1, 2]. Still, their virulence factors are yet to be described. For a first step, we looked for common fungal virulence factors via phenotypic methods. This is a limited approach as the results might vary according to test conditions and negative results do not rule out the presence of the related virulence factor. Nonetheless, when found positive, further studies might be performed to find out the related genes and understand under which conditions they are produced and how they affect pathogenesis. This study checked coagulase, haemolysis, acid proteinase, and phospholipase which were not detectable in any of the isolates with the method used. Esterase positivity was detected *Saprochaete/Magnusiomyces* isolates for the first time. In addition, strong biofilm biomass formation was documented.

Coagulase activity is affected by the type of plasma. Coagulase positivity was reported for *Candida* species, with variations in rabbit, sheep, and human plasma [17–19]. For *Trichosporon*, 5.0% and 27.5% coagulase positivity was detected using human and rabbit plasma, respectively [30]. We used both rabbit and human plasma and evaluated at different time periods but did not observe positivity in *Saprochaete/Magnusiomyces* isolates.

Hemolytic activity of *Saprochaete/Magnusiomyces* isolates was negative on SDA supplemented with 7% sheep or horse blood after 48 h. Variable rates of hemolysis were detected in *Candida* isolates [17]. Although Demir et al. [30] observed hemolysis after 48 of incubation in *Trichosporon*, Sun et al. [31] needed to extend the time period to 96 h. Therefore, extended incubation periods might alter test results for *Saprochaete/Magnusiomyces,* which was a limitation of our study.

Acid proteinases and phospholipases could not be detected in *Saprochaete/Magnusiomyces* isolates in this study. They are important virulence factors for *Candida* with high positivity rates [17, 18]. In another rare yeast, *Trichosporon,* both proteinase and phospholipase activities were negative in different studies [21, 30–32]. However, Pontieri et al. [12] found secreted acid protease in 3 of the 25 *S. capitata* isolates they tested on BSA agar and confirmed the finding by showing the hydrolysis of bovine serum hemoglobin by a spectrophotometer. Although we could not detect acid proteinase positivity, the method used was different from that of Pontieri et al.

Unlike other virulence enzymes, esterase was detected in 56.1% of the *Saprochaete/Magnusiomyces* isolates tested. Esterase positivity is common among *Candida* species and might be positive in almost all *C. albicans* isolates [22]. Moreover, several studies stated 100% esterase positivity in *Trichosporon* [30, 32]. Understanding the molecular basis of esterase production and its contribution to pathogenesis needs further studies for *Saprochaete/Magnusiomyces.*

Surface hydrophobicity is an important factor which affects cell-cell and cell-surface interactions and may alter virulence in medically-significant fungi. As it is directly correlated to adhesion, a shift in biofilm formation ability might be expected, but this output is not confirmed [33]. Of the *Saprochaete/Magnusiomyces* isolates tested, 11 *S. capitata* isolated showed intermediate levels of surface hydrophobicity, with remaining isolates showing low levels. Still, high biofilm-forming ability was present in all isolates, and the effect of surface hydrophobicity on biofilms of *Saprochaete/Magnusiomyces* is yet to be investigated.

Biofilm-forming ability could be demonstrated in all *Saprochaete/Magnusiomyces* isolates all of which showed strong biomass production and metabolic activity using the microplate method. Furthermore, biomass production was higher than the biofilm-positive reference strain *C. albicans* MYA-274 in 40.4% of the isolates. Metabolic activity was mostly slower, with 75.5% of the isolates showing an activity about 25–50% of the positive control, which might be caused by the difference between *Candida* and *Saprochaete/Magnusiomyces*. The growth rate of the latter is generally slower, which might indicate a slower metabolic activity. Biofilm production is an important virulence factor associated with adherence and persistence on surfaces, which contributes to foreign body infections and nosocomial outbreaks. ElGindi et al. [34] speculated that the site of infection is more important than the genus/species of the arthroconidial fungi to predict biofilm-forming capacity. They investigated biofilm formation of clinical and reference strains of yeasts including *S. capitata* CBC 197.35 under fluid flow and commented that the biofilm composition had more filamentous cells, increasing biofilm mass. Moreover, filamentous cells of *S. capitata* (and *Trichosporon asahii*) could resist shear forces due to maintained adherence. Dantonio et al. [13] observed large amounts of slime on the inner lumen of extracted catheters in six *S. capitata* clinical isolates obtained from catheter-related fungemia via scanning electron microscopy (SEM). Kraft et al. [35] visualized the biofilm formation of a *S. clavata* on a titanium-alloy device using SEM. Management strategies for rare yeast infections should be guided by the clinical characteristics and might include options for removing biofilm-harboring tissue and foreign bodies [1, 34]. Removal of all foreign bodies including central venous access devices is recommended for the management of *Saprochaete/Magnusiomyces* infections [1].

We determined MIC values of *Saprochaete/Magnusiomyces* isolates against amphotericin B, fluconazole, voriconazole, and micafungin using two reference microdilution methods. Specific instructions for antifungal susceptibility testing of *Saprochaete/Magnusiomyces* are not available [14, 15]. Still, reference methods developed primarily for *Candida* and *Cryptococcus* are preferred to obtain reliable and reproducible MIC results [1, 14, 15]. An inoculum of

1–5 × 10^6 cell/ml, prepared according to 0.5 McFarland standard, is recommended for *Candida* and *Cryptococcus* for both methods. However, when we prepared *Saprochaete/Magnusiomyces* 0.5 McFarland solutions of 0.08–0.13 optical density and 80–82% transmittance as recommended for *Candida* [26], we could not reach the recommended cell concentrations. Therefore, we ensured the solutions had the required amount of yeast cells by having cell counts and saw that these inocula ranged at 62–66% transmittance. Noster et al. [36] observed that the viable colony counts in inoculum solutions were below the recommended concentration and adjusted them to 0.75 McFarland for antifungal susceptibility testing of *Saprochaete/Magnusiomyces* using the EUCAST method. The need to standardize spectrophotometric measurements to obtain desired cell concentrations was addressed and applied to filamentous fungi previously [37]. As *Saprochaete/Magnusiomyces* cells have variable morphology and volume (Supplementary Figure 3), recommendations for *Candida* may not be appropriate, and further guidance might be needed.

When reading MIC values of *Saprochaete/Magnusiomyces* isolates, despite higher inocula were used, growth was mostly not sufficient in control wells after 24 h of incubation. For testing *Candida* and *Cryptococcus*, the CLSI method encourages reading at 24 h but extends incubation if growth in the control well is not sufficient [14]. The EUCAST method recommends reading after 24 ± 2 h of incubation but states that OD values should exceed 0.2 in the control well before determining MICs [15]. Previous studies also reported MICs after 48 h [5, 8, 38], even if a higher inoculum was used [36]. Achieving sufficient growth in the control wells might pose a problem when determining MICs for *Saprochaete/Magnusiomyces.*

Since clinical breakpoints and epidemiological cutoffs are not available for *Saprochaete/Magnusiomyces,* we only provided MIC values [39, 40]. Determination of MIC values using a reference method is recommended to decide the best management options and accumulate epidemiological data as the susceptibility of individual strains might vary [1]. CLSI and EUCAST microdilution methods mostly give compatible results. In our study, EUCAST MICs had a tendency to be higher than CLSI MICs (except amphotericin B), although variations existed. Fernandez-Ruiz et al. [38] tested three *S. capitata* isolates using CLSI and EUCAST, finding higher MICs with EUCAST, except amphotericin B. Esposto et al. [41] compared EUCAST, CLSI, and Sensititre and reported that MIC_{50} and MIC_{90} values were within one double-dilution. Method-specific breakpoints are recommended to avoid variations of MICs to evaluate the susceptibility of fungal isolates.

For the *Saprochaete/Magnusiomyces* isolates tested, amphotericin B MIC values ranged between 0.5 and 2 µg/ml. Most studies found amphotericin B MICs between 0.5 and 2 µg/ml [1]. But MICs as low as 0.06 µg/ml using CLSI [8] and EUCAST [36] and as high as 8 µg/ml using EUCAST [42] were reported using reference methods in different studies.

Fluconazole MICs were high in *Saprochaete/Magnusiomyces* isolates and were between 0.25 and 16 and 0.5 and 64 µg/ml with CLSI and EUCAST, respectively. High fluconazole MICs were reported in the literature for *Saprochaete/Magnusiomyces*, which mostly ranged between 16 and 32 µg/ml [1, 6, 8, 38, 41]. Still, high doses of fluconazole might be used to treat infections caused by isolates with low fluconazole MICs [1].

Voriconazole MICs were variable for the *Saprochaete/Magnusiomyces* isolates in the study. Although GM was 0.94 µg/ml with EUCAST, a MIC value of > 8 µg/ml was detected in a *S. clavata* isolate, and MICs of 4 µg/ml were detected in six *S. capitata* isolates. CLSI MICs were lower, ranging between 0.016 and 1 µg/ml. Fernandez-Ruiz et al. [38] also detected higher VOR MICs with EUCAST than CLSI, reaching 16 µg/ml in one *S. capitata* isolate. VOR MICs are usually between 0.03 and 0.5 µg/ml in *S. capitata* [1], and the detection of higher MICs in individual isolates is disturbing.

As expected, *Saprochaete/Magnusiomyces* isolates yielded high micafungin MICs. The lowest micafungin MIC was 0.5 µg/ml (EUCAST) at 48 h in a *S. clavata* isolate, which had a MIC of 0.25 µg/ml at 24 h with insufficient growth. High *Saprochaete/Magnusiomyces* echinocandin MICs were also reported in the literature [1, 8, 36]. Echinocandins are not an option for the treatment of *Saprochaete/Magnusiomyces* infections [1]. These results confirmed that this group of antifungals did not have sufficient activity against *Saprochaete/Magnusiomyces*, even in vitro. Individual isolates with low echinocandin (micafungin, caspofungin) MICs were detected in different studies using reference methods [1, 5, 36]. Poor growth might pose a problem when evaluating echinocandin MIC values, as reading at 24 h as recommended might not be reliable due to insufficient growth [14, 15]. Since *Saprochaete/Magnusiomyces* are known to have reduced susceptibility to echinocandins, if encountered, low MICs should be approached with caution and should not be considered as a potential susceptibility.

Saprochaete/Magnusiomyces species are rare human pathogens; therefore, the data on these microorganisms are limited. Recent advances in molecular taxonomy lead to nomenclature changes, and identification in routine microbiology laboratories was revisited. In this study, (1) clinical isolates included in the study might successfully be identified to species level with both MALDI-TOF MS and ID32C supported with conventional tests, as confirmed by sequencing. (2) Common fungal virulence factors were investigated via phenotypic methods as a first step to understand

the pathogenicity of these opportunistic fungi. Coagulase, hemolytic activity, acid protease, and phospholipase could not be detected. However, more than half of the isolates were esterase positive. Surface hydrophobicity was weak in most isolates, but all of them formed biofilms with high biomass production. These results might change according to test conditions but might be a beginning to understand the pathogenesis of *Saprochaete/Magnusiomyces*. (3) Antifungal susceptibility testing methodology needs to be standardized for *Saprochaete/Magnusiomyces*, as spectrophotometric inoculum preparation according to 0.5 McFarland was not adequate and higher (transmittance 62–66%) was suitable. In addition, incubation for 24 h rarely yielded sufficient growth. As MIC values obtained using CLSI and EUCAST were variable, the need to establish method-based epidemiological cutoff values and clinical breakpoints stands.

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Data Availability The datasets generated during and/or analysed during the current study are available at Turkish High Education Council Thesis Center (https://tez.yok.gov.tr/UlusalTezMerkezi/giris.jsp, No: 752412) and from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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