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Research article

Molecular identification and extracellular enzyme production of *Rhodotorula* spp.

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Abstract
Rhodotorula spp. have been associated with human infections, particularly in individuals with weakened immune systems. This study aimed to identify 31 isolates of *Rhodotorula* spp. recovered from coin-operated washing machines. The tubs, lint filters, detergent drawers, and fabric softener drawers were swabbed and streaked on Sabouraud dextrose agar (SDA) supplemented with 0.05 g L^{-1} chloramphenicol. Identification of *Rhodotorula* spp. was performed by using polymerase chain reaction (PCR) with fungus-specific primers targeting the internal transcribed spacer (ITS; ITS1-5.8-ITS2) regions. Moreover, the study investigated the production of extracellular enzymes, including phospholipase, esterase, and proteinase. The results showed that all 31 *Rhodotorula* spp. isolates grew well on SDA, forming round, smooth, moist mucoid, coral-red to salmon-colored colonies at 25 °C within 24 h. PCR analysis yielded approximately 620 bp products, and sequencing analysis identified 25 *Rhodotorula mucilaginosa* (80.65%) and 6 *Rhodotorula dairenensis* (19.35%) isolates. In this study, all *R. mucilaginosa* and *R. dairenensis* showed strong (pz = 0.7–0.76) to very strong phospholipase activity (pz < 0.69). There were 16 *R. mucilaginosa* and 3 *R. dairenensis* that demonstrated esterase activity levels ranging from weak (pz = 0.91) to very strong (pz < 0.69). Only 14 *R. mucilaginosa* isolates displayed proteinase activity levels ranging from weak (pz = 0.92) to very strong (pz < 0.69). Therefore, the results implied that the potential pathogenic fungi distributed in the environment, particularly washing machines, may cause individuals to be at risk of being infected with these opportunistic yeasts, especially immunocompromised patients.

Keywords
Coin-operated washing machines, esterase, phospholipase, proteinase, red yeast

Introduction

Rhodotorula spp. are red or salmon-colored yeast-like fungi that are typically isolated from various natural environments including soil, seawater, freshwater, milk, fruits, and vegetables (Wirth and Goldani, 2012; Chreptowicz et al., 2019; Allahkarami et al., 2021; Šovljanski et al., 2022). Moreover, they can also be recovered from several kinds of household appliances, such as dishwashers, air conditioners, and washing machines, etc. (Kulesza et al., 2021; Pintong et al., 2023; Shiraishi et al., 2023; Zareshahrabadi et al., 2023). Additionally, *Rhodotorula* spp. are part of the human microbiota, residing as commensal fungi in healthy individuals (Caetano et al., 2023). Unfortunately, *Rhodotorula* spp. was previously ignored and considered a non-pathogenic yeast. According to the increased reporting of patients with impaired immune status, HIV infection, central-venous catheter usage, cancer, and hematological malignancies, these conditions contribute to *Rhodotorula* spp. infections (Fung et al., 2009; Spiliopoulou et al., 2012; Kim et al., 2013; Potenza et al., 2019). Moreover, the contamination of this red yeast in hospital environments has been reported (Sanna et al., 2021). Thus, *Rhodotorula* spp. are currently important emerging opportunistic fungi.

Extracellular enzyme production plays a critical role in the pathogenicity of fungi. Phospholipase, an enzyme group comprising A1 (PLA1), A2 (PLA2), B (PLB), C (PLC), and D (PLD), catalyzes the cleavage of ester bonds within phospholipids, which are major constituents of cell membranes (Ghannoum, 2000; Djordjevic, 2010). This hydrolysis by phospholipase facilitates the penetration, invasion, lysis, and injury of host cells (Ghannoum, 2000). Additionally, pathogenic fungi produce esterase or monoacylglycerol lipases, which are lipolytic enzymes (Noori et al., 2017). Furthermore, proteinase production has been observed in various fungi, including *Cryptococcus neoformans* (San Felice) Vuill, *Candida* spp., *Aspergillus fumigatus* (Fresen), and *Scedosporium aurantiacum* (Gilgado, Cano, Gené & Guarro), serving as crucial virulence factors for tissue penetration and invasion (Robinson et al., 1990; Chen et al., 1996; Seifi et al., 2015; Han et al., 2017).

Coin-operated washing machines are household appliances that are currently available in several places such as condominiums, dormitories, apartments, etc. A previous investigation by the authors recovered several kinds of yeasts from coin-operated washing machines, such as *Candida albicans* (C.P. Robin) Berkhout, *Candida tropicalis* (Castell) Berkhout, *Candida parapsilosis* (Ashford) Langeron & Talice, and *Trichosporon* spp., etc. (Pintong et al., 2023). We also isolated yeast-like fungi that displayed red or salmon-colored colonies from these household appliances. Thus, this study aimed to identify *Rhodotorula* spp. using a PCR-based method and evaluate the extracellular enzyme production of *Rhodotorula* spp. isolated from coin-operated washing machines. The findings detail the presence of potentially pathogenic yeasts distributed in household appliances, posing potential health risks, particularly for immunocompromised individuals.

Materials and Methods

Rhodotorula *spp. isolates and DNA extraction*

Thirty-one *Rhodotorula* spp. were originally obtained from our previous investigation of the contamination of fungi in coin-operated washing machines (Pintong et al., 2023). Briefly, tubs, lint filters, detergent drawers, and fabric softener drawers were swabbed with sterile cotton swabs, which were streaked entirely on the surface of Sabouraud dextrose agar (SDA) supplemented with 0.05 g L^{-1} chloramphenicol and incubated at 25 °C for 7 days. Yeast-like fungi that showed red, salmon-

colored colonies were collected and stored at −80 °C for further experimentation. Other yeasts that grew on SDA were also collected for identification, and the results had already been published (Pintong et al., 2023). DNA extraction was performed by growing these red yeasts on SDA and incubating at 25 °C for 24 h. Subsequently, the yeasts were suspended in 100 µl of sterile distilled water and centrifuged at 8,000 rpm at 4 °C for 1 min, after which the supernatant was discarded. Next, 100 µl of 20 mM NaOH was added to the pellet, followed by boiling at 95 °C for 45 min. The suspension was then centrifuged at 12,000 rpm at 4 °C for 10 min, and the resulting supernatant containing genomic DNA was transferred to a new sterile microcentrifuge tube and stored at −20 °C for further experimentation.

Polymerase chain reaction (PCR) and DNA sequencing

The ITS1-5.8S-ITS2 region was amplified using the PCR technique in a total volume of 25 µl, comprising 1 µl of genomic DNA, 12.5 µl of GoTaq® Green Master Mix (Promega, USA), 0.5 µl of each forward primer ITS1 and reverse primer ITS4 (White et al., 1990), and 10.5 µl of distilled water. The PCR reaction was conducted under the following conditions: initial denaturation at 95 °C for 3 min, followed by denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 45 s. The reaction was cycled 35 times, with a final extension step at 72 °C for 5 min. Subsequently, the PCR products were subjected to analysis using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized using a gel documentation system (Bio-Rad). The amplified ITS1-5.8S-ITS2 region was purified and bidirectionally sequenced by 1st BASE DNA sequencing (Apical Scientific Sdn Bhd, Malaysia), and the obtained sequences were edited using BioEdit V7.2.5. These sequences were then compared with existing sequences deposited in GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the resulting sequences were submitted to BankIt for accession numbers.

Analysis of phylogenetic trees and species relationships

The optimal evolutionary model for the ITS datasets was determined using the Bayesian Information Criterion (BIC) within MEGA-X (Kumar et al., 2018). The model with the lowest BIC score was chosen to construct phylogenetic trees via the maximum likelihood method. The percentage of trees in which associated taxa clustered together is indicated adjacent to each branch. Utilizing a matrix of pairwise distances estimated via the maximum composite likelihood approach, a phylogenetic tree comprising our 31 *Rhodotorula* spp. nucleotide sequences and 17 published sequences was generated automatically through a heuristic search, employing the neighbor-joining and BioNJ algorithms. The topology associated with the highest log likelihood value was selected. Branch lengths on the tree correspond to the number of substitutions per site and are drawn to scale. Bootstrap analysis was performed, comprising 1,000 replicates, and the resulting bootstrap values are displayed above the branches. The ITS sequences of 14 *Rhodotorula* spp. published in GenBank were selected based on their classification into the same species and other species of the genus. The ITS sequences of *Symmetrospora coprosmae* NR073317, *C. neoformans* MT310954, and *C. duobushaemulonii* MZ261921 were selected as outgroups.

Preparation of yeast suspension for extracellular enzyme production

Red yeasts were cultivated on SDA and allowed to incubate at 25 °C for 24 h. Subsequently, the yeast cells were adjusted to a turbidity equivalent to 0.5 McFarland standard in 0.89% sterile normal saline. This standardized inoculum suspension was utilized for assessing phospholipase, proteinase, and esterase activities.

Phospholipase activity determination

The production of phospholipase was assessed using an egg yolk medium (Desrini et al., 2023). Egg yolk agar was prepared by combining 13 g SDA, 11.7 g NaCl, 0.11 g $CaCl_2$, and 184 ml distilled water, which was then sterilized by autoclaving. After cooling to 50 °C, 16 ml of sterile egg yolk emulsion (8% v/v) (HiMedia, India) was added to the solution. Aliquots of 5 µl of standard inocula were aseptically spotted onto egg yolk agar plates. Subsequently, these plates were incubated at 25 °C for 7 days, during which precipitation zones around colonies were measured (in mm). The phospholipase activity was determined by calculating the ratio of the colony diameter to the total diameter of the colony plus the precipitation zone. *Candida albicans* ATCC 90028 was used as the positive control. The experiment was conducted in triplicate.

Proteinase activity determination

The assay for proteinase activity utilized yeast carbon-based-bovine serum albumin (YCB-BSA) agar (Treviño-Rangel et al., 2013). A solution containing 1.17% w/v yeast carbon base (HiMedia, India), 0.01% w/v yeast extract (HiMedia, India), and 0.2% w/v bovine serum albumin (Merck, Germany) was prepared, adjusted to pH 5, and sterilized by filtration. This solution was then added to an autoclaved cooled agar solution (50 °C) consisting of 1.5% w/v agar. Ten microliters of the tested red yeast isolates and *C. albicans* ATCC 90028 were inoculated onto YCB-BSA agar plates. The plates were then incubated at 25 °C for 10 days. Following incubation, the plates were stained with 1% w/v amido black and destained with a solution of 30% methanol and 10% acetic acid. Proteolytic activity was indicated by a clear zone around the colony that did not stain with amido black. Proteinase activity was evaluated by calculating the ratio of the colony diameter to the total diameter of the colony plus the clear zone (in mm). The experiment was conducted in triplicate.

Esterase activity determination

The assay for esterase activity employed a Tween 80 opacity test medium (Palmeira et al., 2010). Each liter of the medium contained 10 g Bacto Peptone (HiMedia, India), 5 g NaCl, 0.1 g $CaCl_2$, and 15 g agar, adjusted to pH 6.5, and sterilized by autoclaving. After cooling the medium to approximately 50 °C, 5 ml of Tween 80 (Merck, Germany) was added. Subsequently, 5 µl of inoculum and the control *C. albicans* ATCC 90028 were inoculated onto Tween 80 opacity agar plates and incubated at 25 °C for 10 days. Esterase activity was evaluated by calculating the ratio of the colony diameter to the total diameter of the colony plus the precipitate zone (in mm). The experiment was conducted in triplicate.

Enzymatic activity evaluation

The enzymatic activity of each extracellular enzyme was classified into five groups according to the Pz index. The Pz index was the ratio of the colony diameter to the total diameter of the colony plus

the precipitation zone or clear zone. Pz index: 1 = negative, 0.90–0.99 = weak, 0.80–0.89 = mild, 0.70–0.79 = strong, and < 0.69 = very strong (Treviño-Rangel et al., 2013).

Statistical analysis

The enzymatic activity or Pz index of each extracellular enzyme was computed as the mean and visualized as a heatmap using RStudio version 2023.06.1 + 524 (R Core Team, 2023).

Results

Identification of Rhodotorula *spp. using PCR-based assay*

Thirty-one *Rhodotorula* spp. isolates grew on SDA at 25 °C, forming colonies with characteristic round, smooth, moist, mucoid, coral-red, orange, and salmon-colored appearances (Fig. 1). These red yeast strains were subsequently identified using a PCR-based assay. The PCR products generated from these yeasts were approximately 620 bp in size. Following sequencing, the amplicons were compared with existing data in GenBank using BLASTn, with a similarity cutoff of ≥ 97%. Our analysis revealed that 6 out of 31 isolates were identified as *Rhodotorula dairenensis* (19.35%) (T. Haseg. & I. Banno) Fell, J.P. Samp. & Gadanho, and 25 were identified as *Rhodotorula mucilaginosa* (80.65%) (A. Jörg) F.C. Harrison. The sequences of all *Rhodotorula* spp. isolates were submitted to GenBank, with corresponding accession numbers and isolate numbers detailed in Table 1.

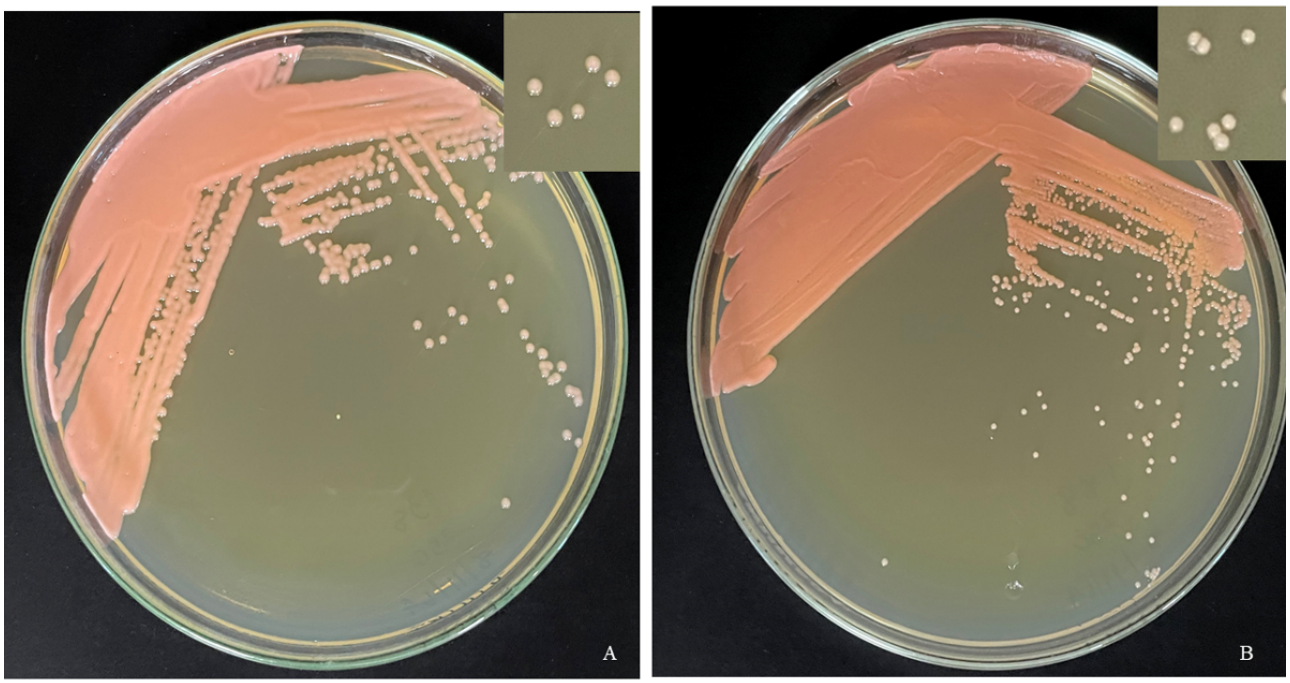

Fig. 1 - Colony morphology of *Rhodotorula* spp. on SDA. A) *R. mucilaginosa* and B) *R. dairenensis* exhibited round, mucoid, coral-red, orange, and salmon hues**.**

Table 1 – *Rhodotorula* spp. isolates and accession numbers.

Yeast species	Isolate number	Accession number
R. mucilaginosa	A2Fb1-023	OQ690178
	A3Fa2-080	OQ690179
	B2F1-095	OQ690180
	C1Fa1-138	OQ690181
	D1Fa2-168	OQ690182
	E2W2-206	OQ690183
	E3Fa2-233	OQ690184
	G1Fa1-304	OQ690185
	G2Fb2-338	OQ690186
	G3W1-347	OQ690187
	G1Fb1-404	OQ690188
	G5W1-432	OQ690189
	H2Fa2-457	OQ690190
	H3Fa1-486	OQ690191
	I1Fa1-525	OQ690192
	I2Fa1-551	OQ690193
	I2Fa2-558	OQ690194
	J2F2-590	OQ690195
	J3F1-600	OQ690196
	K1Fa1-625	OQ690197
	K1Fb1-632	OQ690198
	K2Fa2-651	OQ690199
	K3F1-685	OQ690200
	L1W1-713	OQ690201
	L1Fa2-721	OQ690202
R. dairenensis	A2W1-063	OQ690203
	E1Fa2-188	OQ690204
	H1Fa1-443	OQ690205
	E2Fa1-218	OQ690206
	G3F2-362	OQ690207
	H3Fb1-494	OQ690208

Phylogenetic analysis and species relationships

This study examined the nucleotide sequences of 31 *Rhodotorula* spp., comprising 6 *R. dairenensis* and 25 *R. mucilaginosa*, utilizing a fungus-specific primer ITS. Evolutionary analyses of this region were carried out using MEGA-X (Kumar et al., 2018). The Tamura-Nei (T92) model was employed, incorporating a discrete Gamma distribution (+G) with five rate categories and assuming a fraction of sites to be evolutionarily invariable (+I), a parameter determined based on the best model of evolution analyses (Nei and Kumar, 2000). The BIC score was 7470.788066. Subsequently, a phylogenetic tree was constructed through maximum likelihood analysis, employing the T92 + G + I model with 1,000 bootstrap replications (Fig. 2). Our analysis revealed two distinct genotypes of *R. mucilaginosa*. The first group of *R. mucilaginosa* clustered together with CBS316 (BV = 86), while the second group clustered together with LEMI16 and LEMI17 (BV = 61), forming a sub-clade within the first group. Regarding *R. dairenensis*, all six isolates clustered together with LEMI230 and LEMI2945 (BV = 97), displaying a distinct clade compared to *R. mucilaginosa.*

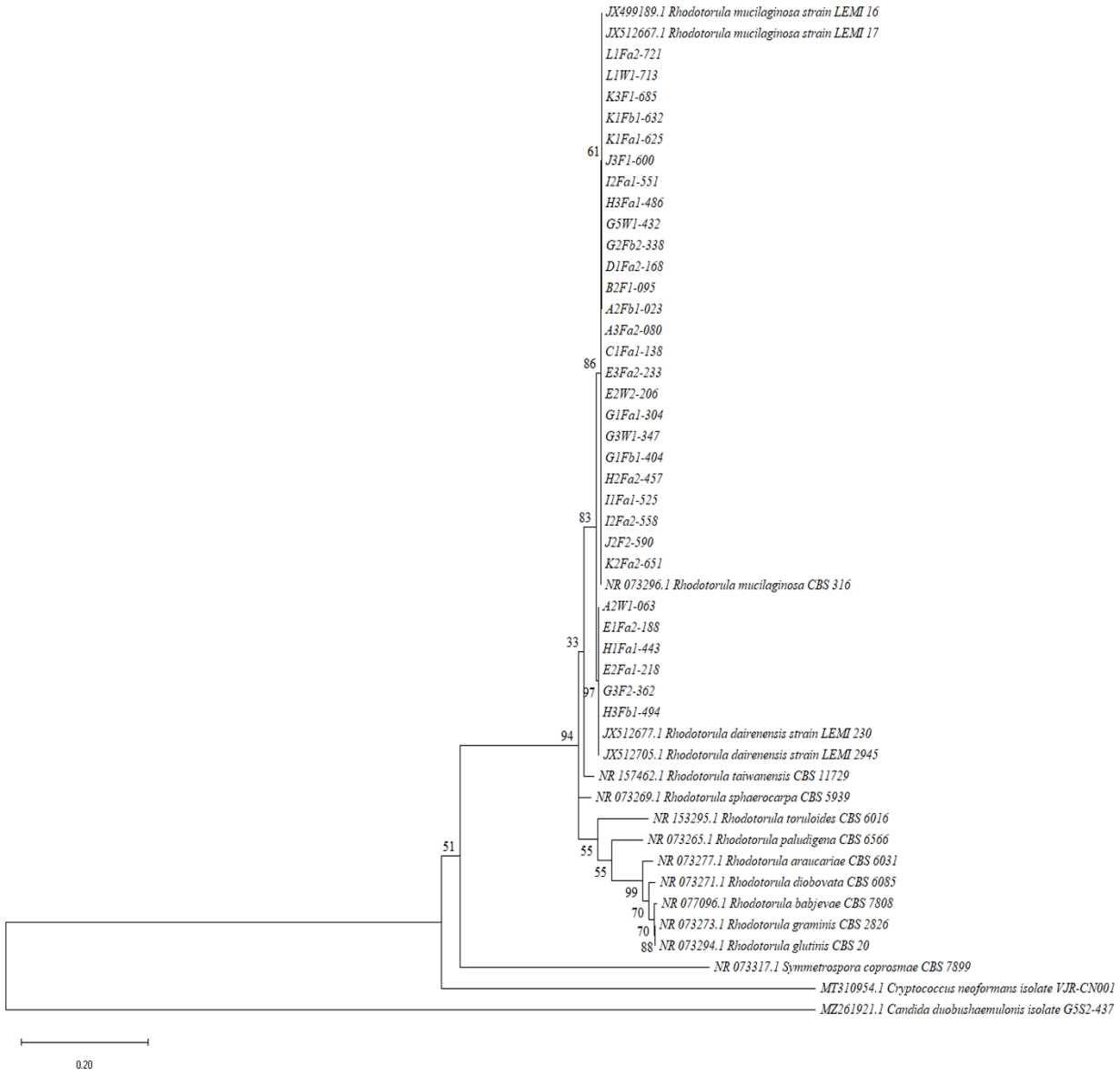

Fig. 2 - Phylogenetic tree constructed from ITS region sequences. The bootstrap values are indicated adjacent to each node. The initial tree was generated automatically using the neighbor-joining and BioNJ algorithms, based on pairwise distance estimates derived from the Maximum Composite Likelihood approach. The topology with the highest log likelihood value was selected. Branch lengths on the tree represent the number of substitutions per site and are drawn to scale.

Extracellular enzyme production

This study investigated the extracellular enzyme production of 31 *Rhodotorula* spp. strains, including *R. mucilaginosa* and *R. dairenensis* (Fig. 3). Enzymatic activity or Pz index was categorized into five groups: 1 = negative, 0.90–0.99 = weak, 0.80–0.89 = mild, 0.70–0.79 = strong, and < 0.69 = very strong (Fig. 4). Initially, all *Rhodotorula* spp. examined in this study exhibited phospholipase activity, ranging from strong (pz = 0.7–0.76) to very strong (pz < 0.69). Furthermore, among the *R. mucilaginosa* isolates, 16 displayed esterase activity ranging from mild (pz = 0.82–0.89) to very strong (pz < 0.69), while 2 *R. dairenensis* isolates exhibited very strong activity and 1 *R. dairenensis* isolate showed weak (pz = 0.91) esterase activity. Regarding proteinase activity, none was observed

in *R. dairenensis*. Conversely, 14 *R. mucilaginosa* isolates exhibited proteinase activity ranging from weak (0.92) to very strong levels. Moreover, three *R. mucilaginosa* isolates (OQ690178, OQ690181 and OQ690184) demonstrated strong to very strong activity in all 3 extracellular enzymes (Fig. 4).

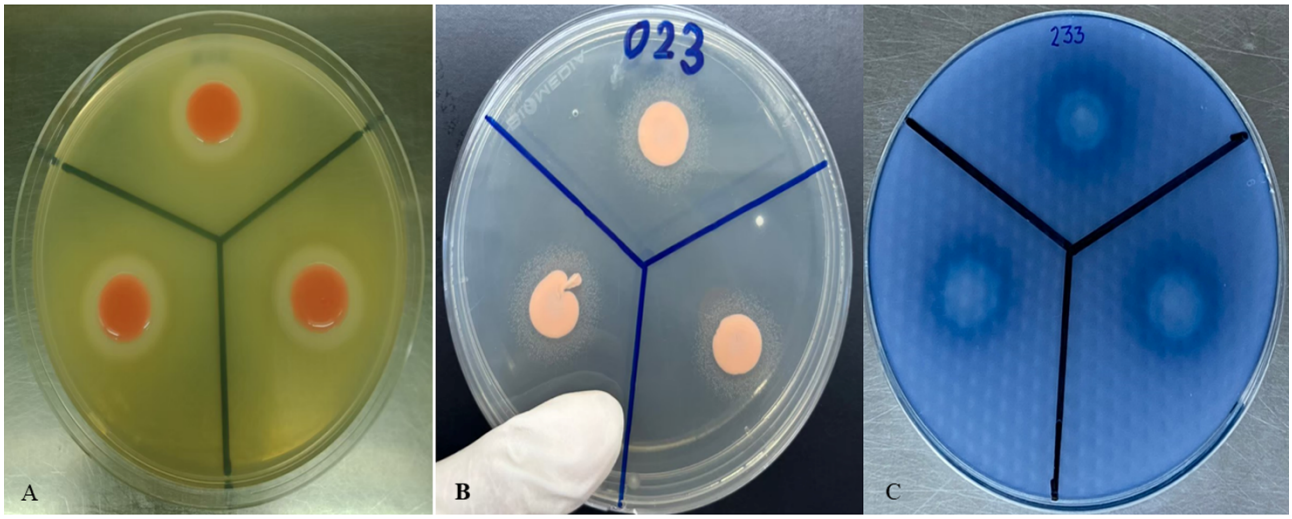

Fig. 3 - Enzymatic activity of *Rhodotorula* spp. with A) representing phospholipase activity indicated by precipitation zones, B) showing esterase activity depicted as a white precipitate, and C) illustrating proteinase activity as a clear zone.

Discussion

Previous studies showed that washing machines were contaminated with plentiful amounts of both filamentous fungi and yeasts, such as *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Mucor* spp., and *Candida* spp. (Dögen et al., 2017; Zareshahrabadi et al., 2023). Our previous study also isolated several kinds of fungi from coin-operated washing machines, particularly *Candida* spp. and *Trichosporon* spp. (Pintong et al., 2023). Moreover, yeast-like fungi that showed red or salmon-colored colonies were also found in the samples. In our investigation, 31 environmental strains of red yeasts were identified as 25 isolates of *R. mucilaginosa* and 6 isolates of *R. dairenensis* using PCR-based techniques targeting the ITS regions. Numerous previous studies demonstrated that *Rhodotorula* spp., *Rhodotorula minuta* (Cif. & Redaelli) F.C. Harrison, *Rhodotorula slooffiae* (E.K. Novák & Vörös-Felkai), and *R. mucilaginosa* were recovered from laundry machines, though data concerning *R. dairenensis* has not been reported (Babič et al., 2015; Nix et al., 2015; Dögen et al., 2017; Zareshahrabadi et al., 2023).

Rhodotorula spp. are saprophytic yeasts commonly found in various environmental sources and are also part of the normal human microbiota, such as respiratory, genital, gastrointestinal, and dermal (Wirth and Goldani, 2012; Allahkarami et al., 2021; Šovljanski et al., 2022; Caetano et al., 2023). The possible reasons for the contamination of these fungi in coin-operated washing machines are the multiple users and the ignorance of the owners of the appliances to maintain and clean them. Interestingly, a previous study recovered *R. mucilaginosa* and *R. slooffiae* from tap water and groundwater, meaning these yeasts could be transferred with water to household appliances (Novak et al., 2016).

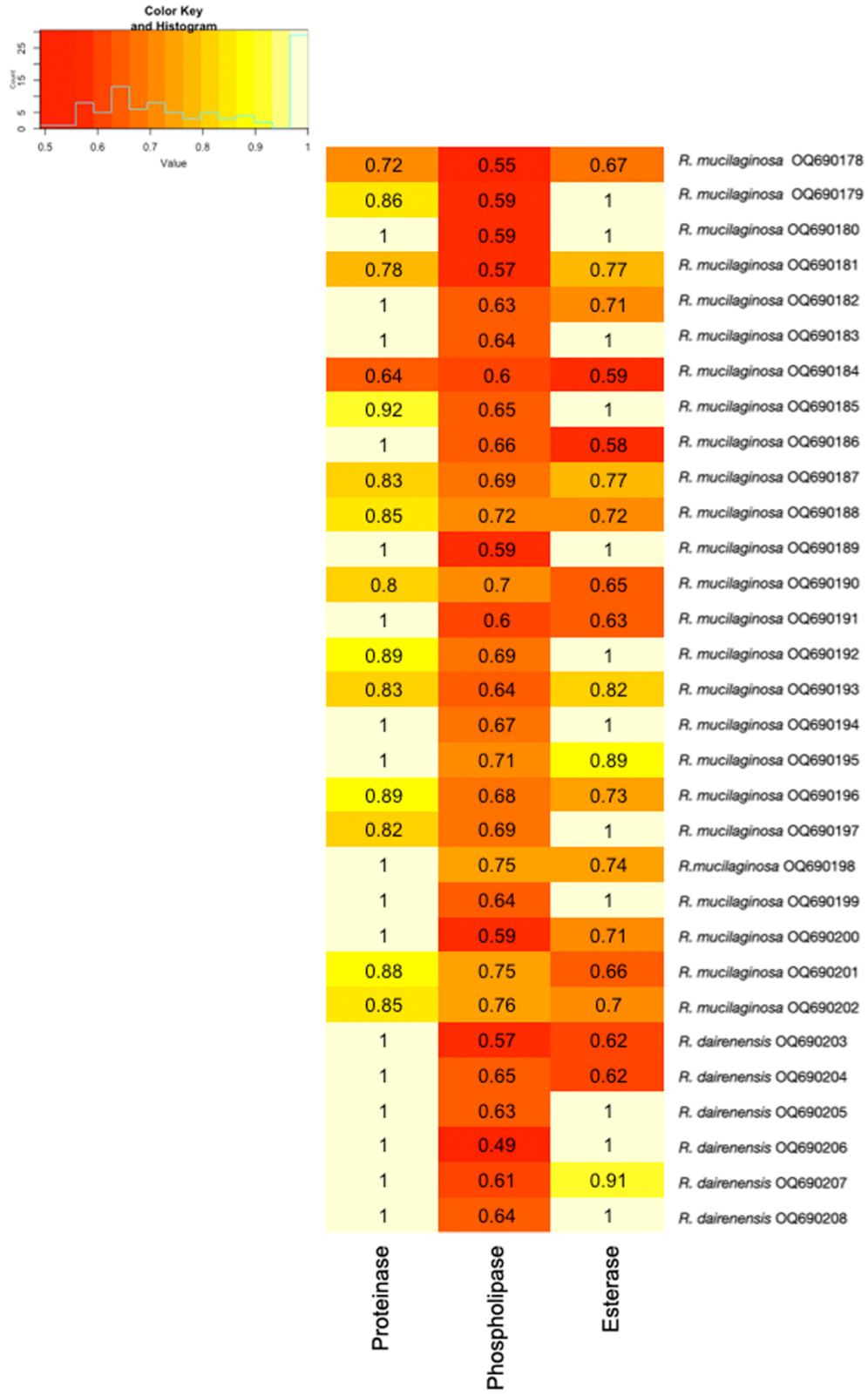

Fig. 4 - A heatmap of extracellular enzymatic activity (phospholipase, esterase, and proteinase) in *R. mucilaginosa* and *R. dairenensis*. The heatmap was generated using RStudio version 2023.06.1 + 524. Enzyme activity was calculated as the mean of the Pz index.

Rhodotorula spp. are traditionally considered nonpathogenic fungi, with the first case of fungemia caused by *Rhodotorula* spp. documented in 1960 (Louria et al., 1960). Currently, *Rhodotorula* spp. infections, particularly *R. mucilaginosa*, are increasingly being reported (Fung et al., 2009; Spiliopoulou et al., 2012; Wirth and Goldani, 2012; Kim et al., 2013). Though *Rhodotorula*spp. infections are mostly caused by *R. mucilaginosa*, *R. dairenensis* have also been reported in cancer patients (Cobo et al., 2020). Phylogenetic tree analysis revealed two distinct genotypes of *R. mucilaginosa*: One closely related to the reference strain *R. mucilaginosa* CBS316, while the other clustered with clinical isolates from Brazil (LEMI16 and LEMI17) (Nunes et al., 2013). Moreover, all six isolates of *R. dairenensis* were grouped with clinical isolates from blood culture samples (LEMI230 and LEMI2945) (Nunes et al., 2013).

Extracellular enzyme production stands as a significant virulence factor among pathogenic fungi, wherein phospholipase and esterase play pivotal roles in penetration and invasion. These enzymes have been identified in various pathogenic yeasts such as *C. neoformans*, *C. albicans*, *C. tropicalis*, and *C. parapsilosis* (Chen et al., 1996; Ghannoum, 2000; Vidotto et al., 2005; Djordjevic, 2010; Pakshir et al., 2013). However, there has been limited exploration of extracellular enzyme production in *Rhodotorula* spp. In the current study, both *R. mucilaginosa* and *R. dairenensis* spp. isolates exhibited phospholipase production, with activities ranging from strong to very strong. Previous research has indicated phospholipase production in *Rhodotorula* spp. isolated from bovine raw milk samples (Melville et al., 2011). Additionally, Seifi et al. (2016) reported phospholipase activity in 82.4% of *Rhodotorula* spp. isolates, with over 50% displaying very strong activity. Nineteen *Rhodotorula* spp. isolates exhibited esterase activity ranging from weak to very strong, while only *R. mucilaginosa* demonstrated weak to very strong proteinase activity. Earlier investigations revealed extracellular enzyme activity, including phospholipase, proteinase, and esterase, in *R. mucilaginosa* isolated from Antarctic permafrost and active layer soils (da Silva et al., 2022).

Although all the *R. mucilaginosa* and *R. dairenensis* isolates in this study were recovered from environmental sources, specifically coin-operated washing machines, some isolates exhibited pathogenic potential based on their extracellular activities. This result implied the presence of potential pathogenic yeasts in the environment, posing potential health risks, especially to immunocompromised individuals. Moreover, the antifungal susceptibility testing, biofilm formation, and genetic relationship between clinical and environmental strains are highlighted for further investigation to provide knowledge of these emerging fungal pathogens.

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References

Allahkarami S, Sepahi AA, Hosseini H, Razavi MR (2021) Isolation and identification of carotenoid-producing *Rhodotorula* sp. from Pinaceae forest ecosystems and optimization of in vitro

carotenoid production. Biotechnology Reports 32:e00687. https://doi.org/10.1016/j.btre.2021.e00687

Babič MN, Zalar P, Ženko B, Schroers HJ, Džeroski S, Gunde-Cimerman N (2015) *Candida* and *Fusarium* species known as opportunistic human pathogens from customer-accessible parts of residential washing machines. Fungal Biology 119(2–3):95–113. https://doi.org/10.1016/j.funbio.2014.10.007

Caetano CF, Gaspar C, Martinez-de-Oliveira J, Palmeira-de-Oliveira A, Rolo J (2023) The role of yeasts in human health: a review. Life (Basel) 13(4):924. https://doi.org/10.3390/life13040924

Chen LC, Blank ES, Casadevall A (1996) Extracellular proteinase activity of *Cryptococcus neoformans*. Clinical and Diagnostic Laboratory Immunology 3(5):570–574. https://doi.org/10.1128/cdli.3.5.570-574.1996

Chreptowicz K, Mierzejewska J, Tkáčová J, Młynek M, Čertik M (2019) Carotenoid-producing yeasts: identification and characteristics of environmental isolates with a valuable extracellular enzymatic activity. Microorganisms 7(12):653. https://doi.org/10.3390/microorganisms7120653

Cobo F, Zaragoza Ó, Navarro-Marí JM (2020) *Rhodotorula dairenensis* fungemia in a patient with cancer. Revista Iberoamericana Micologia 37(2):63–64. https://doi.org/10.1016/j.riam.2020.03.006

da Silva TH, Queres Gomes EC, Gonçalves VN, da Costa MC, Valério AD, de Assis Santos D, Johann S, Convey P, Rosa CA, Rosa LH (2022) Does maritime antarctic permafrost harbor environmental fungi with pathogenic potential? Fungal Biology 126(8):488–497. https://doi.org/10.1016/j.funbio.2022.04.003

Desrini S, Girardot M, Imbert C, Mustofa M, Nuryastuti T (2023) Screening antibiofilm activity of invasive plants growing at the Slope Merapi Mountain, Central Java, against *Candida albicans*. BMC Complementary Medicine and Therapies 23(1):232. https://doi.org/10.1186/s12906-023-04044-2

Djordjevic JT (2010) Role of phospholipases in fungal fitness, pathogenicity, and drug development - lessons from *Cryptococcus neoformans*. Frontiers Microbiology 1. https://doi.org/10.3389/fmicb.2010.00125

Döğen A, Sav H, Gonca S, Kaplan E, Ilkit M, Novak Babic M, Gunde-Cimerman N, de Hoog GS (2017) *Candida parapsilosis* in domestic laundry machines. Medical Mycology 55(8):813–819. https://doi.org/10.1093/mmy/myx008

Fung HB, Martyn CA, Shahidi A, Brown ST (2009) *Rhodotorula mucilaginosa* lymphadenitis in an HIV-infected patient. International Journal of Infectious Diseases 13(1):E27–E29. https://doi.org/10.1016/j.ijid.2008.04.006

Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clinical Microbiology Reviews 13(1):122–143. https://doi.org/10.1128/cmr.13.1.122

Han Z, Kautto L, Nevalainen H (2017) Secretion of proteases by an opportunistic fungal pathogen *Scedosporium aurantiacum*. PLoS One 12(1):e0169403. https://doi.org/10.1371/journal.pone.0169403

Kim HA, Hyun M, Ryu SY (2013) Catheter-associated *Rhodotorula mucilaginosa* fungemia in an immunocompetent host. Infection & Chemotherapy 45(3):339–342. https://doi.org/10.3947/ic.2013.45.3.339

Kulesza K, Biedunkiewicz A, Nowacka K, Dynowska M, Urbaniak M, Stępień Ł (2021) Dishwashers as an extreme environment of potentially pathogenic yeast species. Pathogens 10(4):446. https://doi.org/10.3390/pathogens10040446

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biololgy and Evolution 35(6):1547–1549. https://doi.org/10.1093/molbev/msy096

Louria DB, Greenberg SM, Molander DW (1960) Fungemia caused by certain nonpathogenic strains of the Family Cryptococcaceae. Report of two cases due to *Rhodotorula* and *Torulopsis* glabrata. New England Journal of Medicine 263(25):1281–1284. https://doi.org/10.1056/nejm196012222632504

Melville PA, Benites NR, Ruz-Peres M, Yokoya E (2011) Proteinase and phospholipase activities and development at different temperatures of yeasts isolated from bovine milk. Journal of Dairy Research 78(4):385–390. https://doi.org/10.1017/S0022029911000434

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, New York.

Nix ID, Frontzek A, Bockmühl DP (2015) Characterization of microbial communities in household washing machines. Tenside Surfactants Detergents 52(6):432–440. https://doi.org/10.3139/113.110394

Noori M, Dakhili M, Sepahvand A, Davari N (2017) Evaluation of esterase and hemolysin activities of different *Candida* species isolated from vulvovaginitis cases in Lorestan Province, Iran. Current Medical Mycology 3(4):1–5.

Novak Babič M, Zalar P, Ženko B, Džerosk, S, Gunde-Cimerman N (2016) Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances. Fungal Ecology 20:30–39. https://doi.org/10.1016/j.funeco.2015.10.001

Nunes JM, Bizerra FC, Ferreira RCe, Colombo AL (2013) Molecular identification, antifungal susceptibility profile, and biofilm formation of clinical and environmental *Rhodotorula* species isolates. Antimicrobial Agents and Chemotherapy 57(1):382–389. https://doi.org/10.1128/aac.01647-12

Pakshir K, Zomorodian K, Karamitalab M, Jafari M, Taraz H, Ebrahimi H (2013) Phospholipase, esterase and hemolytic activities of *Candida* spp. isolated from onychomycosis and oral lichen planus lesions. Journal de Mycologie Medicale 23(2):113–118. https://doi.org/10.1016/j.mycmed.2013.04.007

Palmeira VF, Kneipp LF, Alviano CS, dos Santos AL (2010) Phospholipase and esterase production by clinical strains of *Fonsecaea pedrosoi* and their interactions with epithelial cells. Mycopathologia 170(1):31-37. https://doi.org/10.1007/s11046-010-9293-6

Pintong L, Siangwilai R, Wongsuk T, Saibuadaeng P, Khunnarong A, Pumeesat P (2023) Isolation of prominent opportunistic yeasts, *Candida* spp., from coin-operated washing machines. Journal of Microbiology Biotechnology and Food Sciences 12:e5592. https://doi.org/10.55251/jmbfs.5592

Potenza L, Chitasombat MN, Klimko N, Bettelli F, Dragonetti G, Del Principe MI, Nucci M, Busca A, Fracchiolla N, Sciumè M, et al (2019) *Rhodotorula* infection in haematological patient: Risk factors and outcome. Mycoses 62(3): 223–229. https://doi.org/10.1111/myc.12875

R Core Team (2023) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/

Robinson BW, Venaille TJ, Mendis AH, McAleer R (1990) Allergens as proteases: an *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment. Journal of Allergy and Clinical Immunology 86(5):726–731. https://doi.org/10.1016/s0091-6749(05)80176-9

Sanna C, Marras L, Desogus A, Marras B, Montero N, Bertolino G, Schintu M, Coroneo V (2021) Evaluation of *Rhodotorula* spp. contamination in hospital environments. Environmental Monitoring and Assessment 193(3):152. https://doi.org/10.1007/s10661-021-08908-3

Seifi Z, Mahmoudabadi ZA, Hydrinia S, Halvaeezadeh M (2016) Extracellular enzymes production and biofilm formation in *Rhodotorula s*pecies. Current Enzyme Inhibition 12(2):155-160. https://dx.doi.org/10.2174/1573408011666150917175702

Seifi Z, Mahmoudabadi ZA, Zarrin M (2015) Extracellular enzymes and susceptibility to fluconazole in *Candida* strains isolated from patients with vaginitis and healthy individuals. Jundishapur Journal of Microbiology 8(3):e20162. https://doi.org/10.5812/jjm.20162

Shiraishi Y, Harada K, Maeda C, Ogino F, Suzuki Y, Okada N, Tomomatsu K, Sekine Y, Yanagi U, Imanishi T, Ogum T, Asano K (2023) A method to evaluate and eliminate fungal contamination in household air conditioners. Indoor Air 2023:984619. https://doi.org/10.1155/2023/8984619

Šovljanski O, Saveljić A, Tomić A, Šeregelj V, Lončar B, Cvetković D, Ranitović A, Pezo L, Ćetković G, Markov S, et al. (2022) Carotenoid-producing yeasts: selection of the best-performing strain and the total carotenoid extraction procedure. Processes 10(9):1699. https://doi.org/10.3390/pr10091699

Spiliopoulou A, Anastassiou ED, Christofidou M (2012) *Rhodotorula fungemia* of an intensive care unit patient and review of published cases. Mycopathologia 174(4):301–309. https://doi.org/10.1007/s11046-012-9552-9

Treviño-Rangel RdJ, González JG, González GM (2013) Aspartyl proteinase, phospholipase, esterase and hemolysin activities of clinical isolates of the *Candida parapsilosis* species complex. Medical Mycology 51(3):331–335. https://doi.org/10.3109/13693786.2012.712724

Vidotto V, Melhem M, Pukinskas S, Aoki S, Carrara C, Pugliese A (2005) Extracellular enzymatic activity and serotype of *Cryptococcus neoformans* strains isolated from AIDS patients in Brazil. Revista Iberoamericana de Micologia 22(1):29–33. https://doi.org/10.1016/s1130-1406(05)70003-6

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocol: a guide to methods and applications (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). San Diego, CA, USA: Academic Press, pp 315–322.

Wirth F, Goldani LZ (2012) Epidemiology of Rhodotorula: an emerging pathogen. Interdisciplinary Perspectives on Infectious Diseases 2012:1–7. https://doi.org/10.1155/2012/465717

Zareshahrabadi Z, Amirzadeh N, Pakshir K, Nasr R, Mohammadi R, Zomorodian K (2023) Evaluation of building washing machines as an extreme environment for potentially pathogenic fungi. Scientific Report 13(1):21408. https://doi.org/10.1038/s41598-023-48598-0