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# Role of the Random Amplified Polymorphic DNA Detection in Typing *Malassezia pachydermatis* Strains from Neonatal Intensive Care Unit (NICU) Patients' Clinical Isolates

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## Abstract

*Malassezia* spp. has been recognized among neonatal intensive care unit (NICU) patients' commensals and pathogens, accounting for a significant number of invasive fungal infections. The Random Amplified Polymorphic DNA (RAPD) may be used for *Malassezia* spp. strains typing from clinical isolates, demonstrating high resolution and specificity. Herein, we propose a retrospective analysis of *Malassezia* spp. isolates, aiming to investigate their identity and transmission pathways. Moreover, we documented *Malassezia* spp. prevalence within the University Hospital Policlinico of Catania, Italy. The analysis collected a total number of 16 *M. pachydermatis* and categorized them into four different clusters, hypothesizing a horizontal transmission. Although the essential role of microbiological sample cultures, our data suggested further environmental surveillance protocols to prevent NICU patients' colonization due to the *Malassezia* spp. persistence and adhesion within healthcare surfaces.

**Keywords:** *Malassezia* spp.; NICU; colonization; PCR-RAPD; microbiological surveillance



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## 1. Introduction

Yeast species are recognized as the most important aetiological agent of fungal infections among critical patients, especially regarding intensive care units (ICU) [1]. ICU patients have significant risk factors, such as prolonged corticosteroid therapies and length of stay, central venous catheters, and exposure to broad-spectrum antibiotics (e.g., third-generation cephalosporins) [1]. These risk factors are emphasized among neonatal intensive care unit (NICU) patients, including parenteral nutrition, prematurity, immunological impairments, and histamine-receptor antagonist administration [2,3].

Moreover, preterm birth (<1500 g), surgery, endotracheal external devices, low birth weight, and gestational age of less than 32 weeks contribute to extensive fungal colonization and potential invasive infections [3].

Literature documented invasive fungal infection epidemiology among NICUs, reporting *Candida* spp. and *Malassezia* spp. as the main pathogens [4–9]. On one hand,

*Candida* spp. has a consolidated role among the fungal aetiological agents, accounting for well-known morbidity percentages and hospital settings diffusion [4–7]. On the other hand, *Malassezia* spp. infection rates and epidemiology may have been underestimated during the past decades. Both these fungal genera usually integrate the human microbiota, occasionally expressing their opportunistic attitude among fragile patients [4–9]. Despite their conventional presence within the skin and/or mucosal microbiota, *Malassezia* spp. and *Candida* spp. have been identified in the case of bloodstream or deep-seated fungal infections. Some studies revealed that *Malassezia* spp. invasive infections are sometimes more prevalent (2.1%) than candidaemia episodes (1.4%) within NICU settings [9]. The same scientific data demonstrated the presence of *Malassezia* spp. on NICU surfaces and healthcare personnel's hands, along with blood culture isolation [10].

After the first recognition of *Malassezia* spp. as a fungemia causative yeast (1981) [11], colonization surveillance protocols have been applied to demonstrate this microorganism's presence within NICU patients reporting parenteral nutrition or external venous devices [12,13]. Consequently, cessation of intravenous lipid nutrition and catheter removal are the best clinical practice in managing *Malassezia* spp.—invasive infections, together with evidence-based treatments due to underestimated infection rates [14]. Unfortunately, most *Malassezia* species often require a specific lipid source to grow, contributing to difficult isolation within laboratory diagnostic workflows. According to this assumption, some diagnostic protocols include lipid-enriched culture media (e.g., Dixon agar) or direct molecular investigations on biological samples [15].

*Malassezia furfur* and *Malassezia pachydermatis* represent the most common isolated species in the case of systemic infections. Despite *M. furfur* being commonly reported as a human commensal and pathogen, *M. pachydermatis* first emerged from animal sources [16]. This species has not colonized neonates since the early weeks after the preterm birth, but its colonization may be related to neonates' closeness with personnel or relatives reporting animals' contacts [16].

Molecular methods demonstrated high sensitivity and negative predictive values in diagnosing severe fungal infections caused by different aetiological agents [17,18].

Furthermore, *M. pachydermatis'* non-lipid-dependent nature simplifies isolation procedures, allowing molecular assay usage on grown colonies [19]. This species often persists on incubator surfaces and human hands after conventional cleansing protocols [20]. Despite the recent refinement of sequencing analysis techniques, typing methods mainly involved the PCR Random Amplified Polymorphic DNA (PCR-RAPD), a fingerprinting method extensively used to study *M. pachydermatis* strains' homologies or differences [21,22].

Herein, we propose a retrospective analysis of *Malassezia* spp. isolates, aiming to investigate *M. pachydermatis* identity through PCR-RAPD and demonstrate a potential homogeneous transmission pathway for this species. Another fundamental purpose was to integrate scientific data about *Malassezia* spp. epidemiology within critical healthcare settings, documenting its prevalence in our hospital.

## 2. Materials and Methods

A one-year (January 2021–January 2022) retrospective analysis included all the *M. pachydermatis* strains isolated from NICU patients from the University Hospital Policlinico of Catania. We decided to focus our attention on that specific period because it documented an overall increase in opportunistic infections within our hospital.

The isolates emerged during a routine surveillance protocol, including urine, oropharyngeal swabs, stool samples, and gastric aspirates. Clinicians implemented gastrointestinal surveillance procedures through gastric aspirates according to previously published scientific data [22]. Despite the invasive attitude of these samples' collection, they demonstrated

a sensitivity higher than rectal swabs and reflected the upper gastrointestinal tract, unlike stool samples [23]. Blood cultures integrated the microbiological diagnostic confirmation only after clinicians' evaluation of a systemic infection's clinical suspicion. All these surveillance procedures were applied due to previous *Malassezia* spp. fungemia cases within preterm newborns. The analysis did not directly involve human beings, focusing only on microbial strains from their clinical isolates. Furthermore, the study did not require supplementary biological samples. We analyzed clinical strains from surveillance specimens after the conventional diagnostic routine. On the premise of these assumptions, an ethical committee statement was not necessary.

Figure 1 describes the applied surveillance protocol furnishing the analyzed clinical isolates.

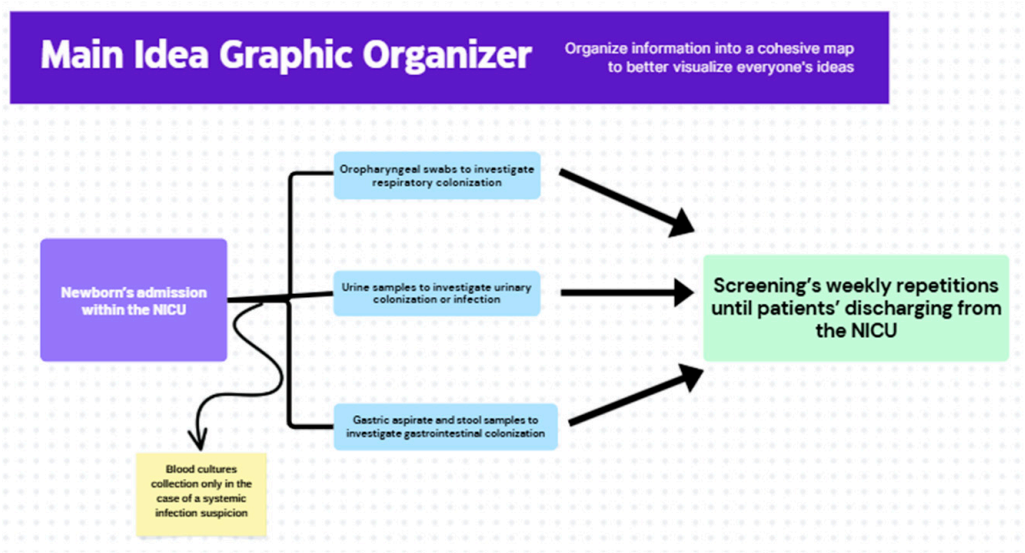


**Figure 1.** Summary of the routine surveillance protocol that furnished clinical samples and *M. pachydermatis* isolates for experimental analysis.

### *2.1. Culture and Identification*

Although they have a non-lipid-dependent attitude, all the isolates grew on a *Malassezia* (Dixon's) Selective Agar (Vakutest Kima, Arzergrande, Italy). All the isolates were identified through mass spectrometry from the colonies' direct spots. We adopted the MALDI Biotyper® Sirius System (Bruker, Billerica, MA, USA) technology, basing the identification on the MBT IVD Library (June 2021 Doc. No. 5023016).

### *2.2. Extraction Protocols*

The molecular typing included all the identified isolates along with the *M. pachydermatis* ATCC 14522 control strain. We applied ultra-pure water washing and SET buffer (20 mM Tris pH 8, 30 mM EDTA, 80 mM NaCl) dilution on grown *Malassezia* spp. cells according to previous documented extraction protocols [24]. The extracted nucleic acid underwent a dilution with 100 µL of 10 mM Tris–HCl pH 8.0, and 1 mM EDTA containing 20 µg/mL of RNAse A [24]. Finally, an automated extraction protocol through the Nuclisens Easymag (bioMérieux Italia, Bagno a Ripoli, Firenze, Italy) was utilized to obtain the fungal DNA according to the manufacturer's instructions [25].

### *2.3. PCR Random Amplified Polymorphic DNA (PCR-RAPD)*

The extraction processes gathered a DNA concentration equal to 25 ng. The subsequent amplification phase used the VeritiTM Thermocycler (Applied Biosystems, ThermoScientific, Waltham, MA, USA), which was set according to Williams et al.'s previous investiga-

tions [23,24]. We integrated the following primers' sequences: OPA02: 5′-TGCCGAGCTG-3′; OPA04: 5′-AATCGGGCTG-3′; P3: 5′-GTAGACCCGT-3′ [26]. The amplified genomes were placed into an agarose gel (1.8%) with TBE O.5X (54 g/L Tris Base, 27.5 g of boric acid, and Ethylenediaminetetraacetic acid 0.5 mM, with a definitive pH = 8). The same TBE solution was used to perform an electrophoresis (1 h, 100 V). We compared the DNA fragments' length to a 50 bp marker (New England BioLabs, Ipswich, MA, USA). We reproduced 3 times all the PCR cycles to verify the RAPD-PCR reproducibility and ensure a sufficient number in validating the entire process.

The definitive analysis was performed through a transilluminator (Uvitec, Cambridge, UK) and sequence analyzer software (Ailunce HD2, v1.14, Ailunce, Shenzhen, China). The genetic distance between the different samples was evaluated through a binary matrix, indicating 1 for the presence of specific reproducible bands and 0 for their absence. We elaborated a dendrogram using the Jaccard index and Sneath and Sokal's UPGMA (Unweighted Pair Group Method with Arithmetic Average) method.

## 3. Results

Among 264 microbiological surveillance samples from 88 NICU patients, 46 (17.4%) positive cultures emerged. Specifically, 36 results (78.2.%) reported *Malassezia* spp. isolation. Otherwise, only 10 positive cultures (3.8%) revealed *Candida* spp. Among *Candida* spp. positive results, we identified five (50%) *Candida albicans*, four (40%) *Candida parapsilosis*, and one (10%) *Candida glabrata* strain. All these isolates represented respiratory or gastrointestinal colonization. As regards *Malassezia* spp. positive cultures, we identified 20 (55.5%) *M. furfur* and 16 (44.4%) *M. pachydermatis* strains. *M. furfur* reported three blood culture-positive results, while *M. pachydermatis* did not cause systemic infections.

Focusing on *M. pachydermatis* isolates, the analysis collected a total of 16 *M. pachydermatis* isolates from 11 patients (Table 1). Four isolates (1, 2, 3, 4) belonged to patient 1, while two isolates were derived from patient 8 (strains 11, 12) and patient 10 (strains 14, 15). The isolates emerged from urine, stool, and gastric aspirate samples, representing colonization episodes.

Specifically, the microscopic examination did not reveal any neutrophils along with the absence of clinical urinary symptoms, while stool and gastric aspirate samples documented a gastrointestinal colonization.

The primer's usage allowed the revelation of different bands, whose number and type concurred to define polymorphisms and genetic profiles. The OPA02 primer produced a different band number depending on the analyzed strains. Specifically, isolates 16 gathered 2 bands, strains from 1 to 9 obtained 6 bands, and strains from 10 to 15 collected 7 bands. All the isolates belonging to patient 1 (1, 2, 3, 4) demonstrated the same genetic identity. Patients 2 to 7 reported a single *M. pachydermatis* isolate, which had identical genetic profiles. On the other hand, the single isolate belonging to patient 11 reported a specific polymorphism profile. The OPA04 primer registered two different polymorphisms (5 bands and 6 bands). This analysis identified the same genetic profiles for all the strains belonging to patient 1 and the strains deriving from patient 4. Finally, the P3 primer produced two bands for each strain, identifying a single polymorphism for all the involved microorganisms. Figures 2–4 show graphical details about the reported genetic profiles through the different used primers.

**Table 1.** Details on patients and biological samples for all the included *M. pachydermatis* strains.

| Patients | Isolation Data | Strain Number | Urine | Stool | Gastric Aspirate |
|---|---|---|---|---|---|
| Patient 1 | 10 May 2021 | 1 | | *M. pachydermatis* | |
| | 9 August 2021 | 2 | *M. pachydermatis* | | |
| | 19 October 2021 | 3 | *M. pachydermatis* | | |
| | 21 October 2021 | 4 | | *M. pachydermatis* | |
| Patient 2 | 18 June 2021 | 5 | | *M. pachydermatis* | |
| Patient 3 | 12 July 2021 | 6 | | *M. pachydermatis* | |
| Patient 4 | 24 June 2021 | 7 | | *M. pachydermatis* | |
| Patient 5 | 10 September 2021 | 8 | | *M. pachydermatis* | |
| Patient 6 | 16 September 2021 | 9 | *M. pachydermatis* | | |
| Patient 7 | 3 June 2021 | 10 | *M. pachydermatis* | | |
| Patient 8 | 16 October 2021 | 11 | | *M. pachydermatis* | |
| | 19 October 2021 | 12 | | | *M. pachydermatis* |
| Patient 9 | 5 October 2021 | 13 | | *M. pachydermatis* | |
| Patient 10 | 7 October 2021 | 14 | | *M. pachydermatis* | |
| | 5 November 2021 | 15 | *M. pachydermatis* | | |
| Patient 11 | 30 January 2022 | 16 | *M. pachydermatis* | | |

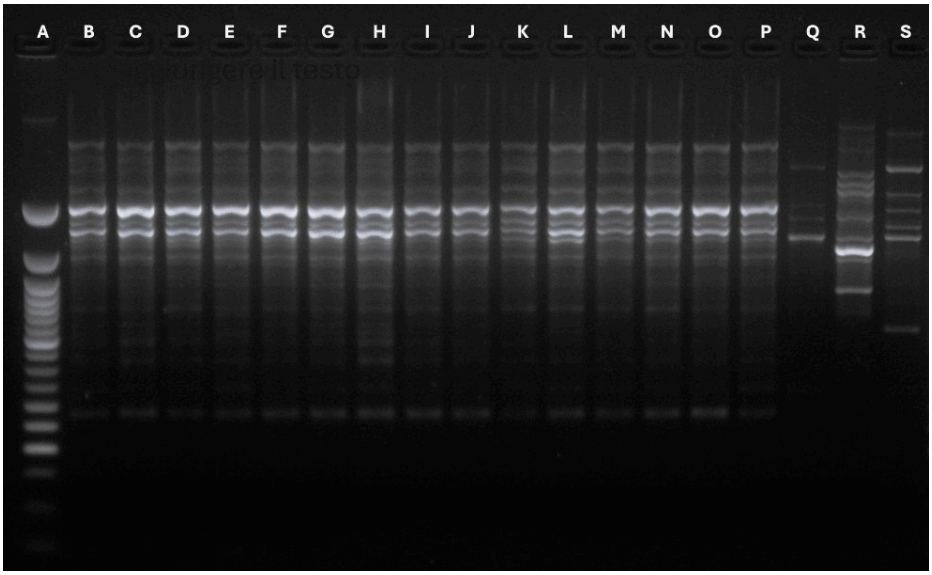


**Figure 2.** Graphical details about the genetic profiles gathered through the OPA02 primer. **A**: Ladder 50 bp; **B**: Strain 1-Stool; **C**: Strain 2-Urine; **D**: Strain 3-Urine; **E**: Strain 4: Stool; **F**: Strain 5-Stool; **G**: Strain 6-Stool; **H**: Strain 7-Stool; **I**: Strain 8-Stool; **J**: Strain 9: Urine; **K**: Strain 10-Urine; **L**: Strain 11-Stool; **M**: Strain 12-Gastric aspirate; **N**: Strain 13: Stool; **O**: Strain 14: Stool; **P**: Strain 15-Urine; **Q**: Strain 16-Urine; **R**: *Malassezia globosa* Control strain; **S**: ATCC *Malassezia pachydermatis* CD69.

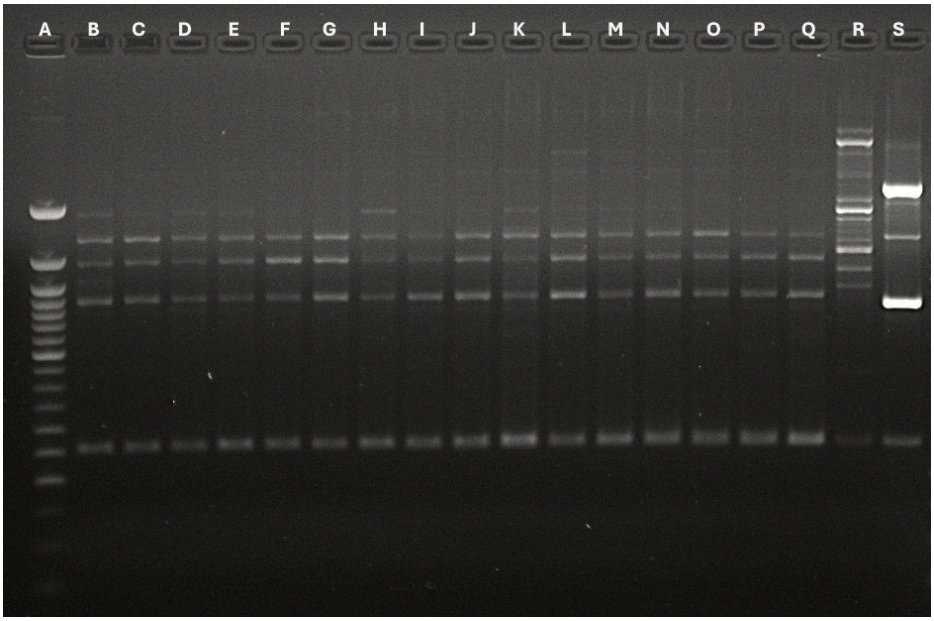


**Figure 3.** Graphical details about the genetic profiles gathered through the OPA04 primer. **A**: Ladder 50 bp; **B**: Strain 1-Stool; **C**: Strain 2-Urine; **D**: Strain 3-Urine; **E**: Strain 4: Stool; **F**: Strain 5-Stool; **G**: Strain 6-Stool; **H**: Strain 7-Stool; **I**: Strain 8-Stool; **J**: Strain 9: Urine; **K**: Strain 10-Urine; **L**: Strain 11-Stool; **M**: Strain 12-Gastric aspirate; **N**: Strain 13: Stool; **O**: Strain 14: Stool; **P**: Strain 15-Urine; **Q**: Strain 16-Urine; **R**: *Malassezia globosa* Control strain; **S**: ATCC *Malassezia pachydermatis* CD69.

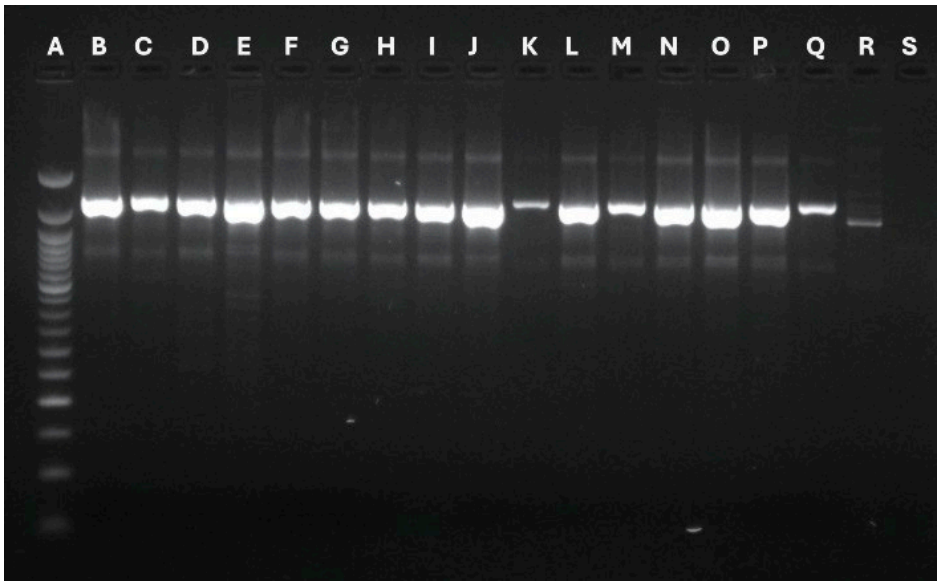


**Figure 4.** Graphical details about the genetic profiles gathered through the P3 primer. **A**: Ladder 50 bp; **B**: Strain 1-Stool; **C**: Strain 2-Urine; **D**: Strain 3-Urine; **E**: Strain 4: Stool; **F**: Strain 5-Stool; **G**: Strain 6-Stool; **H**: Strain 7-Stool; **I**: Strain 8-Stool; **J**: Strain 9: Urine; **K**: Strain 10-Urine; **L**: Strain 11-Stool; **M**: Strain 12-Gastric aspirate; **N**: Strain 13: Stool; **O**: Strain 14: Stool; **P**: Strain 15-Urine; **Q**: Strain 16-Urine; **R**: *Malassezia globosa* Control strain; **S**: ATCC *Malassezia pachydermatis* CD69.

The polymorphism analysis contributed to defining four *Malassezia* spp. clusters, along with a specific isolation period within the NICU setting. A dendrogram (Figure 5) described four clusters for the analyzed *M. pachydermatis* strains (Figure 2).

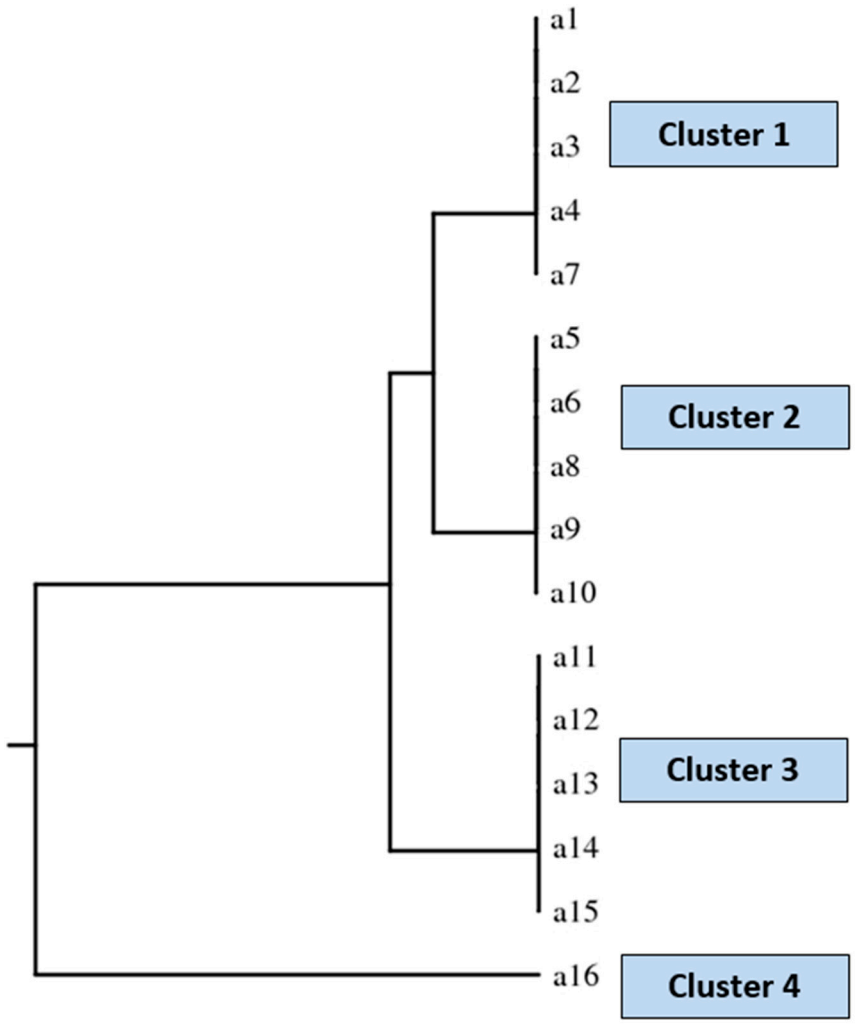


**Figure 5.** Dendrogram describing the identified four clusters. Cluster 1 includes strains number 1, 2, 3, 4 (Patient 1), and 7 (Patient 4). Cluster 2 contains strains number 5 (Patient 2), 6 (Patient 3), 8 (Patient 5), 9 (Patient 6), and 10 (Patient 7). Cluster 3 includes strains number 11 and 12 (Patient 8), 13 (Patient 9), and 14 and 15 (Patient 10). Finally, cluster 4 includes strain number 16 (Patient 11).

## 4. Discussion

The spread of *Malassezia* spp. in colonization and infection episodes poses a significant healthcare challenge among ICU patients, particularly neonates [24–33]. While *M. furfur* is frequently isolated, there have been a few documented cases of systemic infections caused by *M. pachydermatis* in intensive care patients. The limited pathogenic role of *M. pachydermatis* suggests that these infection cases depend heavily on the patients' fragile conditions. According to our data, *Malassezia* spp. was the main colonizing fungal species during the study period. We focused our analysis on *M. pachydermatis* to investigate its potential modes of transmission within a critical hospital environment.

Previous studies have described various molecular methods for typing *Malassezia* spp. RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) demonstrated a discrete discriminatory power, distinguishing strain groups with low-cost and slow procedures. Pulsed-field gel electrophoresis (PFGE) proposed standardized protocols and guaranteed a significant identification power. Unfortunately, this technique requires dedicated personnel, high costs, and prolonged procedures [25]. Furthermore, fingerprinting models represented a historical and functional tool in typing *Malassezia* spp. strains, furnishing elevated reproducibility rates. This old procedure is regrettably marked by a relevant technical complexity when compared to the RAPD [26]. On the other hand,

modern whole-genome sequencing (WGS) techniques revealed the maximum analysis quality, identifying clonal relationships and phylogenetic features at high confidence levels [27]. Moreover, WGS produces wide-spectrum lineage-specific details and may discover new genes or mobile elements related to precise microorganisms' characteristics [27]. Despite the high precision and specificity in defining *Malassezia* spp. genome and polymorphisms, the significant lipid content within this yeast's cell wall can lead to enzyme inhibition during extraction and interfere with DNA purification. Additionally, the genomic complexity can create artifacts due to repetitive sequences and variable regions. These characteristics make it challenging to standardize sequencing methodologies for *Malassezia* spp. [28–36].

We employed the RAPD method to confirm the genetic identity of all included strains, as it offers more standardized protocols and requires less expertise in a diagnostic laboratory routine. This technology demonstrated significant capabilities for strain discrimination and identification, defining specific clusters based on identified polymorphisms and precise isolation periods within the analyzed hospital ward.

Specifically, four heterogeneous clusters emerged during different isolation periods: cluster 1 included isolates from May 2021 to June 2021; cluster 2 comprised strains from June 2021 to September 2021; cluster 3 contained isolates from October 2021 to November 2021; and cluster 4 included isolates from January 2022.

Despite the separation of clusters, all the isolates were likely transmitted horizontally via healthcare personnel's hands, which had previous contact with animals. We hypothesized that such transmission may relate to *M. pachydermatis'* animal origin, similar to *M. furfur*, whose colonization in neonates may result from exposure to vaginal mycobiome during natural deliveries. We could not confirm this hypothesis and aim to further investigate *Malassezia* spp. strains within the same hospital unit in the coming months.

Additionally, the potential role of transmission during breastfeeding and contact with neonates' relatives may be examined in future studies. Previous data have indicated a potential genomic link between *Malassezia* spp. isolated from neonates and their mothers during the intensive care recovery period, emphasizing the necessity of maternal care [37].

Regardless of the identified species, our experimental analysis underscores the importance of implementing surveillance strategies in critical neonatal settings. Unfortunately, the study had a limited sample size and included few pathogenic species, necessitating future expansion to involve more patients and isolates of *Malassezia* spp. Multi-center studies, including prospective analysis, may be planned in the near future to confirm our hypotheses. Similar studies should probably include hand-sampling and environmental surveillance procedures to confirm the primary *Malassezia* spp. sources.

Despite noted colonization by *Malassezia* spp., the NICU patients included in this study did not develop any systemic infections, likely due to prompt prophylactic measures and appropriate patient management within the healthcare unit. The isolation of colonizing species has led to increased sanitization of surfaces and healthcare workers' hands. Furthermore, positive surveillance cultures have led to a systematic and planned distribution of further surveillance procedures through fungal cultures within the NICU.

Systemic infections documented were attributed to *M. furfur*, prompting considerations for future typing analyses of this species, which demonstrates significant pathogenic relevance in critical human hosts. Furthermore, scientific data indicate *M. pachydermatis'* ability to survive on surfaces inside newborn incubators, highlighting the importance of controlling the entire neonatal recovery environment [28,29,34–39].

Although microbiological cultures from biological samples play a crucial role, the above-mentioned studies have led to hypotheses concerning the implementation of environmental surveillance protocols to prevent NICU patients' colonization from personnel contacts or surface adherence.

The colonization of environmental surfaces and human anatomical regions by *Malassezia* spp. is associated with potential infection cases among patients with prolonged hospital stays.

These considerations may prompt further investigations into the cost-effectiveness of microbiological surveillance. Future analyses could explore economic aspects such as complications from severe fungal infections and the associated costs of prolonged hospitalization.

## 5. Conclusions

*Malassezia* spp. revealed surviving and colonizing attitudes within healthcare-related environments, potentially causing severe infections. Consequently, surveillance protocols should always include *Malassezia* spp. specific isolation conditions. However, this fungal genus requires specific growth conditions and nutritive elements. Our findings underscore that specific infection-control procedures may prevent and contain *Malassezia* infections, documenting this microorganism's prevalence and presence within critical settings. Appropriate microbiological media and incubation periods are essential due to the identification difficulties associated with this particular yeast [39]. Once identification workflows for *Malassezia* spp. are established, microbiological surveillance may become a fundamental strategy in documenting fungal colonization and possible infection outcomes. Punctual surveillance protocols may enrich the overall epidemiological knowledge about specific yeast species, enhancing their potential pathogenic role within critical hospital settings.

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**Institutional Review Board Statement:** We analyzed clinical strains from surveillance specimens after the conventional diagnostic routine. On the premise of these assumptions, an ethical committee statement was not necessary.

**Informed Consent Statement:** In compliance with GDPR (EU 2016/679) and Italian Legislative Decree 196/2003 (as amended by Legislative Decree 101/2018), irreversibly anonymized biological samples are not considered personal data, and informed consent is not required.

**Data Availability Statement:** All the gathered data are included within the manuscript. The raw data supporting the conclusions of this article will be made available by the authors on request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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