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Ex Vivo Fungal Nail Penetration Study: Effects of Causative Organisms, Nail Polish and Age

Kanyalak Munprom | Sumanas Bunyaratavej | Penvadee Pattanaprichakul | Pattriya Jirawattanadon | Lalita Matthapan | Waranyoo Prasong | Chatisa Panyawong | Akkarapong Plengpanich | Charussri Leeyaphan

Department of Dermatology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Correspondence: Charussri Leeyaphan (charussrilee@gmail.com)

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ABSTRACT

Background: Few ex vivo studies have investigated the virulence factors of fungi causing onychomycosis. The effect of nail polish in predisposing or protecting against onychomycosis remains debatable.

Objectives: This ex vivo study aimed to identify the nail invasion ability of dermatophytes, non-dermatophytes and yeast, with and without nail polishing, in the nails of young and elderly individuals.

Methods: Six fungal species were tested: dermatophytes (*Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*), non-dermatophytes (*Neoscytalidium dimidiatum*, *Fusarium* spp.) and *Candida albicans*. Nail plates from eight volunteers (four aged ≥ 70 years; four aged < 70 years) were divided into polished and non-polished groups, incubated with each fungus and evaluated at 2, 4 and 8 weeks. Positive results were determined the presence of fungal hyphae or pseudohyphae penetrating the nail plate, with the enlargement of invasive fungal elements confirmed by histology.

Results: At 2 weeks, *N. dimidiatum* exhibited the highest nail invasion rate (15/16, 93.75%), whereas *C. albicans* showed the lowest (1/16, 6.25%). Fungal penetration into nail plates increased with longer incubation durations. At 8 weeks, *C. albicans* did not invade any polished nail plates; however, the difference in invasion rates between polished and unpolished nail plates was not statistically significant. Additionally, age did not significantly affect the invasion of most fungi in this ex vivo study.

Conclusions: This ex vivo study supported the concept that fungal virulence is the main determining factor for nail invasion. *N. dimidiatum* caused the most and fastest nail plate penetration. Nail polishing may slow the penetration of low-virulence organisms.

1 | Introduction

Onychomycosis, the common nail disorder in clinical practice, is caused by dermatophytes, non-dermatophytes and yeasts [1, 2]. The common causative agents especially in tropical countries are *Trichophyton rubrum* and *T. mentagrophytes*, followed by non-dermatophyte moulds such as *Neoscytalidium dimidiatum* [1–5]. Yeasts such as *Candida albicans* can also cause onychomycosis, especially in individuals with wet work exposure or immunocompromised hosts [6]. Each fungus has a different type of nail infection based on its virulence. However, the virulence of each fungus in nail penetration, including the depth and speed of penetration, has rarely been studied.

Nail polishing is a popular cosmetic, especially among younger females. Currently, it is debatable whether nail polish can aid in the prevention of onychomycosis. Some experts suggest that nail polish may increase the risk of nail infections due to fungal

contamination in polish bottles [7]. Despite being benign, onychomycosis can negatively impact a patient's quality of life, social interactions and self-esteem [1]. To hide the appearance of damaged nails, some patients apply nail polish. A literature review revealed no previous ex vivo studies demonstrating the ability of fungal invasion in nail plate with or without polishing by histopathology.

In addition to factors related to infectious pathogens, host factors can contribute to the development of onychomycosis. Risk factors include older age, diabetes mellitus, immunosuppression and peripheral vascular disease [1, 2, 8]. Older individuals are more prone to fungal nail infections and are less likely to achieve a complete cure. A previous study revealed that patients aged ≥ 70 years tended to have lower rates of complete onychomycosis cure than those aged < 70 years [9].

An ex vivo study can help determine the factors influencing fungal nail infection. Thus, this study aimed to identify the factors affecting the capacity of dermatophytes, non-dermatophytes and yeasts to invade nail plates, including age and nail polishing, via ex vivo histologic examination.

2 | Materials and Methods

2.1 | Ethical Approval

This ex vivo study was approved by the Siriraj Institutional Review Board (Si053/2023).

2.2 | Sample Collection and Preparation

A total of 12 nail plates were collected from 8 healthy volunteers. Six fingernails from each volunteer, with the longest free edges, were selected and collected twice, with a 2-month interval, from the same finger. Four participants were aged ≥ 70 years, and the remaining four were younger than 70 years. Nails were clipped with a width of 4 mm from the edge of each nail. In each age group, nail plates were further divided into nail-polished and non-polished groups. For the nail-polished group, each nail plate was painted on both sides with transparent nail polish composed mainly of ethyl acetate, nitrocellulose, acetyl tributyl citrate and cellulose acrylate butyrate.

2.3 | Baseline Examination

At baseline, the nail plate was cut, with a portion of the nail ground for 20% potassium hydroxide examination and culture to exclude infection. A sample was excluded from the study if either potassium hydroxide or fungal culture was positive for fungus at baseline.

2.4 | Fungal Inoculation

Each nail plate was divided into six segments and sterilised in an autoclave. The segments were then inoculated with six fungal species: dermatophytes (*T. rubrum*, *T. mentagrophytes*,

TABLE 1 | Nail penetration results for each fungal species after 2, 4 and 8 weeks of incubation.

Duration (weeks)	Penetration rate	Total $n = 16$ per fungus (%)					
		Candida albicans	*Trichophyton mentagrophytes*	*Trichophyton rubrum*	*Microsporum canis*	*Fusarium* spp.	*Neoscytalidium dimidiatum*
2	Negative	15 (93.75)	5 (31.25)	9 (56.25)	9 (56.25)	11 (68.75)	1 (6.25)
	< 1/3 of nail	1 (6.25)	7 (43.75)	5 (31.25)	5 (31.25)	5 (31.25)	7 (43.75)
	≥ 1/3 of nail	0	4 (25)	2 (12.5)	2 (12.5)	0	8 (50)
4	Negative	15 (93.75)	3 (18.75)	8 (50)	2 (12.5)	6 (37.5)	0
	< 1/3 of nail	1 (6.25)	5 (31.25)	6 (37.5)	8 (50)	7 (43.75)	6 (37.5)
	≥ 1/3 of nail	0	8 (50)	2 (12.5)	6 (37.5)	3 (18.75)	10 (62.5)
8	Negative	14 (87.5)	3 (18.75)	7 (43.75)	0	3 (18.75)	0
	< 1/3 of nail	2 (12.5)	4 (25)	5 (31.25)	6 (37.5)	4 (25)	3 (18.75)
	≥ 1/3 of nail	0	9 (56.25)	4 (25)	10 (62.5)	9 (56.25)	13 (81.25)

TABLE 2 | Comparison of nail penetration results by age and polish for each fungus species.

Fungi	Number of nails				Total
	Age < 70 years non-polished	Age < 70 years polished	Age ≥ 70 years non-polished	Age ≥ 70 years polished	
Candida albicans					
Week 2					
Negative	3/4	4/4	4/4	4/4	15/16
Penetrate < 1/3 of nail	1/4	0	0	0	1/16
Penetrate ≥ 1/3 of nail	0	0	0	0	0
Week 4					
Negative	3/4	4/4	4/4	4/4	15/16
Penetrate < 1/3 of nail	1/4	0	0	0	1/16
Penetrate ≥ 1/3 of nail	0	0	0	0	0
Week 8					
Negative	2/4	4/4	4/4	4/4	14/16
Penetrate < 1/3 of nail	2/4	0	0	0	2/16
Penetrate ≥ 1/3 of nail	0	0	0	0	0
Trichophyton mentagrophytes					
Week 2					
Negative	0	1/4	2/4	2/4	5/16
Penetrate < 1/3 of nail	3/4	2/4	1/4	1/4	7/16
Penetrate ≥ 1/3 of nail	1/4	1/4	1/4	1/4	4/16
Week 4					
Negative	0	1/4	2/4	0	3/16
Penetrate < 1/3 of nail	2/4	0	0	3/4	5/16
Penetrate ≥ 1/3 of nail	2/4	3/4	2/4	1/4	8/16
Week 8					
Negative	0	1/4	2/4	0	3/16
Penetrate < 1/3 of nail	2/4	0	0	2/4	4/16
Penetrate ≥ 1/3 of nail	2/4	3/4	2/4	2/4	9/16

(Continues)

TABLE 2 | (Continued)

Fungi	Number of nails				Total
	Age < 70 years non-polished	Age < 70 years polished	Age ≥ 70 years non-polished	Age ≥ 70 years polished	
Trichophyton rubrum					
Week 2					
Negative	3/4	1/4	2/4	3/4	9/16
Penetrate < 1/3 of nail	1/4	2/4	1/4	1/4	5/16
Penetrate ≥ 1/3 of nail	0	1/4	1/4	0	2/16
Week 4					
Negative	3/4	1/4	2/4	2/4	8/16
Penetrate < 1/3 of nail	1/4	2/4	1/4	2/4	6/16
Penetrate ≥ 1/3 of nail	0	1/4	1/4	0	2/16
Week 8					
Negative	3/4	1/4	2/4	1/4	7/16
Penetrate < 1/3 of nail	1/4	2/4	1/4	1/4	5/16
Penetrate ≥ 1/3 of nail	0	1/4	1/4	2/4	4/16
Microsporum canis					
Week 2					
Negative	2/4	2/4	3/4	2/4	9/16
Penetrate < 1/3 of nail	1/4	2/4	0	2/4	5/16
Penetrate ≥ 1/3 of nail	1/4	0	1/4	0	2/16
Week 4					
Negative	0	0	0	2/4	2/16
Penetrate < 1/3 of nail	3/4	3/4	0	2/4	8/16
Penetrate ≥ 1/3 of nail	1/4	1/4	4/4	0	6/16
Week 8					
Negative	0	0	0	0	0
Penetrate < 1/3 of nail	2/4	2/4	0	2/4	6/16
Penetrate ≥ 1/3 of nail	2/4	2/4	4/4	2/4	10/16

(Continues)

TABLE 2 | (Continued)

Fungi	Number of nails				Total
	Age < 70 years non-polished	Age < 70 years polished	Age ≥ 70 years non-polished	Age ≥ 70 years polished	
Fusarium spp.					
Week 2					
Negative	3/4	3/4	2/4	3/4	11/16
Penetrate < 1/3 of nail	1/4	1/4	2/4	1/4	5/16
Penetrate ≥ 1/3 of nail	0	0	0	0	0
Week 4					
Negative	2/4	2/4	2/4	0	6/16
Penetrate < 1/3 of nail	2/4	1/4	2/4	2/4	7/16
Penetrate ≥ 1/3 of nail	0	1/4	0	2/4	3/16
Week 8					
Negative	0	2/4	1/4	0	3/16
Penetrate < 1/3 of nail	2/4	1/4	0	1/4	4/16
Penetrate ≥ 1/3 of nail	2/4	1/4	3/4	3/4	9/16
Neoscytalidium dimidiatum					
Week 2					
Negative	0	0	0	1/4	1/16
Penetrate < 1/3 of nail	2/4	2/4	2/4	1/4	7/16
Penetrate ≥ 1/3 of nail	2/4	2/4	2/4	2/4	8/16
Week 4					
Negative	0	0	0	0	0
Penetrate < 1/3 of nail	1/4	2/4	1/4	2/4	6/16
Penetrate ≥ 1/3 of nail	3/4	2/4	3/4	2/4	10/16
Week 8					
Negative	0	0	0	0	0
Penetrate < 1/3 of nail	1/4	1/4	0	1/4	3/16
Penetrate ≥ 1/3 of nail	3/4	3/4	4/4	3/4	13/16

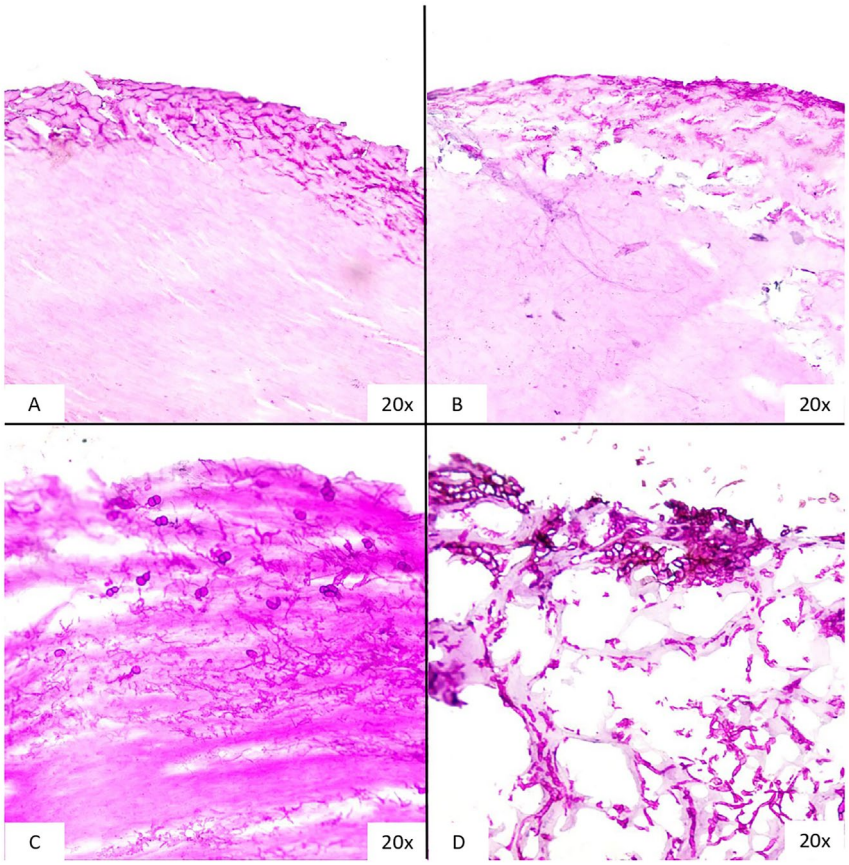

FIGURE 1 | Examples of histological examination of fungal penetration into clipped nails. (A) Penetration of *Trichophyton mentagrophytes* less than 1/3 of the nail thickness (age ≥ 70 years, without nail polish, at week 8); (B) Penetration of *Microsporum canis* less than 1/3 of the nail thickness (age < 70 years, with nail polish, at week 4); (C) Penetration of *Fusarium* spp. greater than or equal to 1/3 of the nail thickness (age ≥ 70 years, with nail polish, at week 8); (D) Penetration of *Neoscytalidium dimidiatum* greater than or equal to 1/3 of the nail thickness, with nail keratin destruction (age < 70 years, with nail polish, at week 8).

Microsporum canis), non-dermatophytes (*N. dimidiatum*, *Fusarium* spp.) and yeast (*C. albicans*). The fungi used in this investigation were strains isolated from patients diagnosed with fungal skin or nail infections. The inoculated nail plates were incubated for 2, 4 and 8 weeks at 26°C.

2.5 | Histologic Examination

After the nail plates were incubated with the fungus, 20% potassium hydroxide was applied to soften the nails. The nail plates were then fixed in 10% formalin and stained with periodic acid–Schiff for histopathological examination. Two independent dermatopathologists performed histologic examinations. In cases of disagreement, a consensus-based discussion was conducted. A positive result was determined by the presence of fungal hyphae or pseudohyphae penetrating the nail plate.

3 | Results

3.1 | Fungal Penetration Over Time

The results of nail penetration are shown in Tables 1 and 2. Fungal penetration was classified into three categories: negative results (no penetration), penetration less than one-third of the nail thickness (Figure 1A,B), and penetration equal to or greater than one-third of the nail thickness (Figure 1C,D). Deeper fungal penetration was detected with longer incubation durations. Fungal penetration was observed in 46/96 (47.9%) nail plates at 2 weeks, increasing to 62/96 (64.6%) at 4 weeks and 69/96 (71.9%) at 8 weeks of incubation. At 2 weeks, *N. dimidiatum* penetrated the nail plates the most (93.75%), whereas *C. albicans* invaded the nail plates the least (6.25%). At 8 weeks, only 12.5% of the nail plates incubated with *C. albicans* showed penetration. In contrast, *N. dimidiatum* penetrated all the nail plates (100%) after 4 weeks. Moreover, *N. dimidiatum* had the greatest proportion of nail plates with deep fungal invasion ≥ 1/3 of the nail thickness at 2 weeks (50%), 4 weeks (62.5%) and 8 weeks (81.25%).

3.2 | Effect of Nail Polish on Fungal Penetration

The effect of nail polish on fungal penetration is shown in Figure 2. *C. albicans* did not invade any polished nail plates during the 8-week study period. *N. dimidiatum* penetrated the nail plates the most in both non-polished and polished group at 2 weeks (100% vs. 87.5%, respectively). Furthermore, *N. dimidiatum* was found to have the greatest number of nail plates

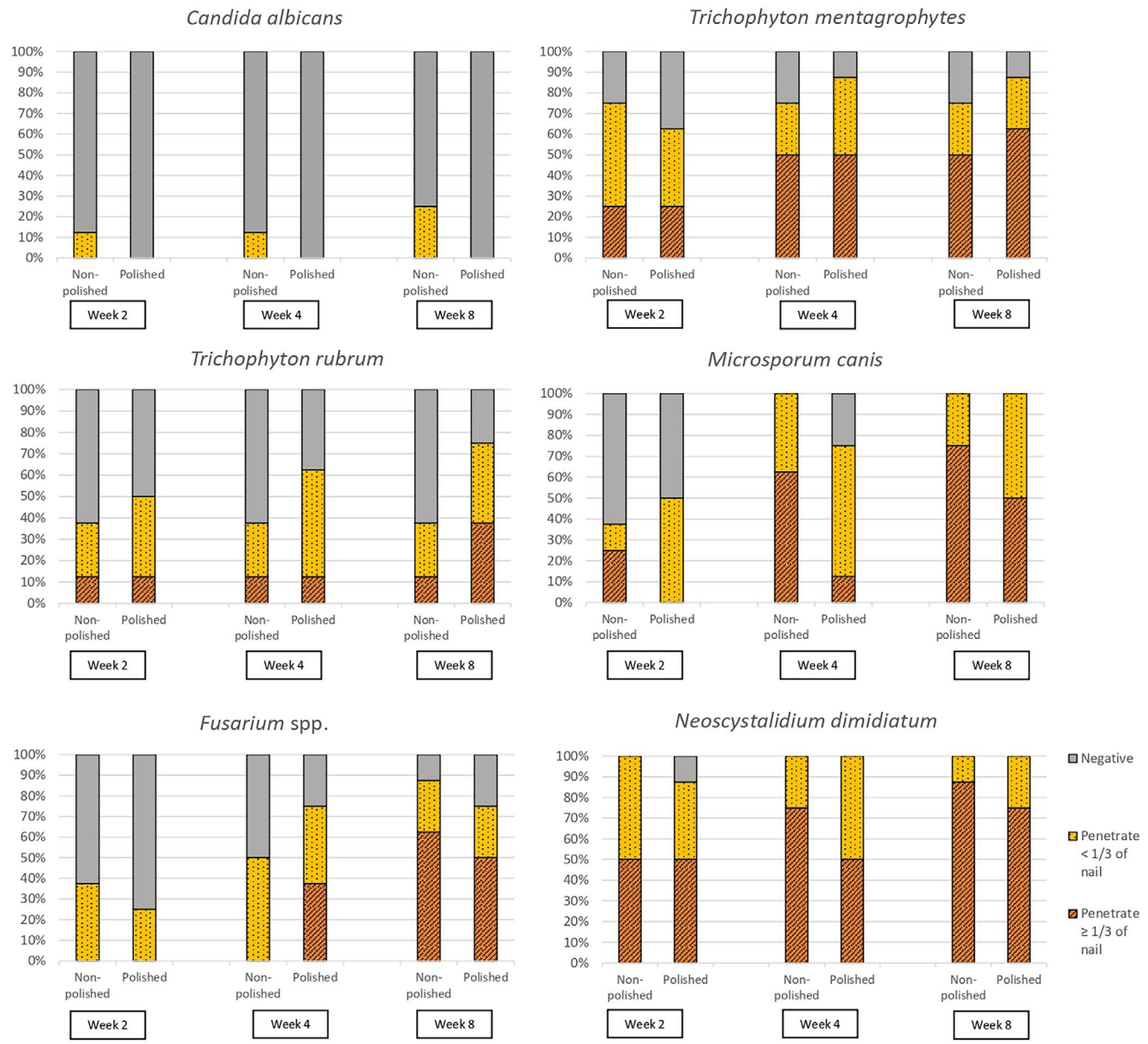

FIGURE 2 | Comparison of fungal nail penetration between polished and non-polished nails after 8 weeks of incubation.

with deep fungal invasion ≥1/3 of the nail thickness in both non-polished and polished group in 4 weeks (75% vs. 50%, respectively) and 8 weeks (87.5% vs. 75%, respectively) of incubation. However, nail penetration by all fungi was not significantly different between non-polished and polished nail plates.

3.3 | Influence of Age on Fungal Penetration

Overall, nail invasion did not significantly differ between the age groups in this study (Figure 3). After 8 weeks, *C. albicans* was not detected invading most nail plates. However, for all fungi except *T. mentagrophytes* and *C. albicans*, nail plates from the older age group presented greater numbers of penetrated nail plates and greater fungal penetration than those from the younger age group did at week 8. There were no significant differences in nail invasion between polished and non-polished nail plates in the young and elderly subgroups.

4 | Discussion

This pilot ex vivo study evaluated factors affecting onychomycosis. One benefit of using an ex vivo study is that the results cannot be influenced by confounding variables such as underlying medical conditions. Thus, an ex vivo study can be utilised to assess factors associated with fungal penetration in nail plates. An additional advantage is the ability of an ex vivo study to assess fungal virulence on the basis of penetration timing and depth. The fungi's penetration depth increased with prolonged incubation. The penetration of higher-virulence fungi can be detected in early incubation periods.

Neoscytalidium dimidiatum penetration was observed as early as 2 weeks of incubation, confirming its severe virulence in invading nails and producing onychomycosis. In contrast, *C. albicans* penetration was detected the least and took longer to penetrate the nail plate. *M. canis* typically infects hair and skin

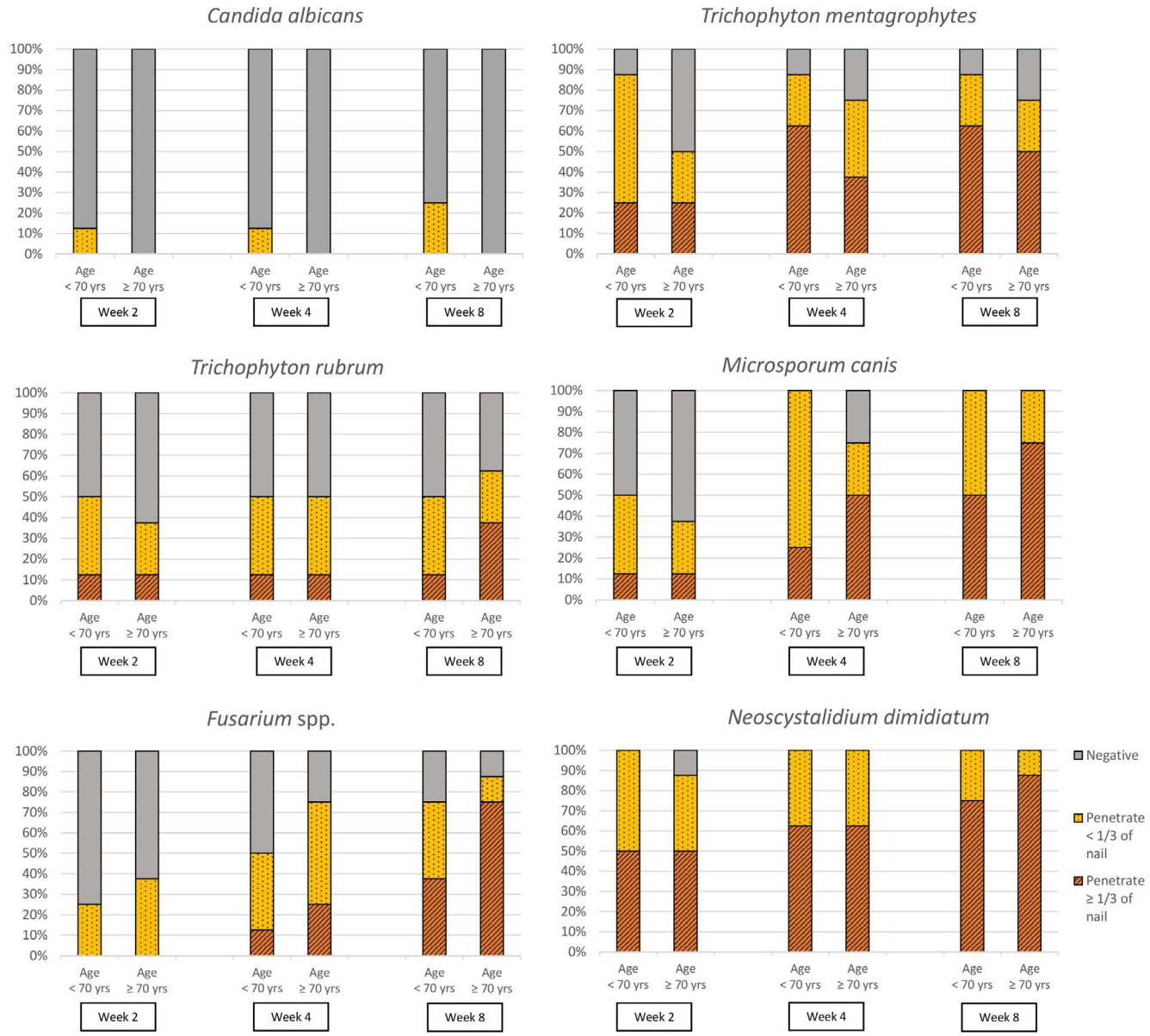

FIGURE 3 | Comparison of fungal nail penetration between age groups (< 70 years and ≥ 70 years) after 8 weeks of incubation.

but rarely nails due to its poor affinity for nail keratin [10, 11]. However, *M. canis* can still affect nails, especially in immunocompromised hosts, individuals with concurrent tinea corporis, and those with a history of pet exposure [10, 12]. This ex vivo study revealed that *M. canis* can penetrate nail keratin, supporting its ability to cause onychomycosis.

Surprisingly, fungal penetration into nail plates was unaffected by age in this ex vivo study. The nails were taken from healthy patients, and the experimental environment was regulated. These findings may imply that the nail structure in young and elderly individuals is not a predisposing factor for onychomycosis, but the immune system under the nail and the nail growth rate may play more important roles in pathogenesis. In real life, elderly individuals mostly have comorbidities that affect nail fragility, including diabetes mellitus, peripheral arterial disease and impaired renal function [9, 13, 14]. Furthermore, elderly people typically have slower-growing nails. These factors increase the risk of developing onychomycosis [13].

Reports on the association between nail polish and onychomycosis are scarce. However, some experts recommend avoiding nail polish in patients with onychomycosis. Nail polishing can cause contact dermatitis, leading to onycholysis, paronychia and onychia, which facilitate fungal infection [13]. Furthermore, a previous study demonstrated the viability of fungi in nail polish due to contamination of the polish bottles, which may increase the risk of onychomycosis [7]. The present ex vivo investigation revealed that nail polishing tends to slow the penetration of some fungi, including *C. albicans*. However, nail polish did not have a protective effect on most fungi. Thus, it could be inferred that appropriate nail polish can shield the nail from infection by low-virulence fungi, but it cannot prevent onychomycosis caused by high-virulence fungi. However, to evaluate

the impact of nail polish on onychomycosis, a clinical study may be needed to demonstrate real-life situations such as nail polish and wet work.

This study had several limitations. Because this was a pilot study, a small sample of nail plates was investigated. Furthermore, additional in vivo research is still needed because the study was conducted ex vivo. All nail plates used in this study were autoclaved using the same method to control for potential confounding factors that could influence nail penetration. In future studies, the sterilisation process should be adjusted to minimise structural changes.

In conclusion, this study confirmed the severe virulence of *N. dimidiatum* in invading nail plates. Age had little effect on the ability of fungi to penetrate nail plates ex vivo, whereas nail polishing had an effect on slowing the penetration of fungi, especially those with low virulence.

Author Contributions

Kanyalak Munprom: conceptualization, methodology, investigation, formal analysis, visualization, writing – original draft, writing – review and editing. **Sumanas Bunyaratavej:** conceptualization, methodology, investigation, project administration, supervision, writing – review and editing, visualization. **Penvadee Pattanaprichakul:** conceptualization, investigation, visualization, project administration, methodology, supervision, writing – review and editing, resources. **Pattriya Jirawattanadon:** conceptualization, methodology, investigation, visualization, project administration, supervision, writing – review and editing. **Lalita Matthapan:** conceptualization, investigation, methodology, visualization, writing – review and editing, writing – original draft, resources, formal analysis. **Waranyoo Prasong:** conceptualization, methodology, investigation, writing – original draft, visualization, writing – review and editing, resources, formal analysis. **Chatisa Panyawong:** conceptualization, methodology, investigation, writing – review and editing, visualization, resources. **Akkarapong Plengpanich:** conceptualization, methodology, investigation, visualization, writing – review and editing, resources. **Charussri Leeyaphan:** conceptualization, methodology, visualization, writing – review and editing, resources, formal analysis, validation, investigation, project administration, writing – original draft, supervision.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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