

MOLECULAR PROFILING REVEALS A DIVERSE ORAL MYCOBIOME IN HEAD AND NECK CANCER PATIENTS FROM PAKISTAN

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ABSTRACT

Head and neck cancer (HNC) is a prevalent malignancy in Pakistan, with treatments like radiotherapy and chemotherapy increasing the risk of oral fungal infections. This study aimed to characterize the diversity of oral yeast species in HNC patients in Pakistan using a combination of phenotypic and molecular diagnostic techniques.

Saliva samples were collected from 110 HNC patients at KIRAN Hospital and 100 healthy controls. Yeasts were isolated on culture media and initially screened using two chromogenic media: Brilliance Candida Agar (BCA) and CHROMagar™ Candida Plus (CCP). Molecular identification was performed by amplifying the Internal Transcribed Spacer (ITS) region, followed by Restriction Fragment Length Polymorphism (RFLP) analysis with the *MspI* enzyme and DNA sequencing for definitive speciation. Phylogenetic analysis and statistical modeling were conducted to understand species relationships and risk factors.

A total of 126 yeast isolates were recovered from HNC patients. Chromogenic media identified up to six *Candida* species, with CCP showing superior differentiation. Molecular methods revealed a greater diversity of 11 yeast species. DNA sequencing confirmed the presence of common pathogens like *Candida albicans*, *C. tropicalis*, and *Rhodotorula mucilaginosa*, as well as uncommon species such as *Cyberlindnera fabianii* (reported for the first time in Pakistan in this context) and *Candida gattii*. PCR-RFLP was useful for common species but could not differentiate *C. albicans* from *C. dubliniensis* or species lacking an *MspI* restriction site. Statistical analysis confirmed that HNC patients had a significantly higher risk (OR: 19.94-174.48, $p < 0.05$) and diversity of yeast colonization compared to healthy controls. This study highlights a complex oral mycobiome in Pakistani HNC patients, with species diversity underestimated by conventional methods. While chromogenic media offer a rapid screening tool, DNA sequencing of the ITS region remains the gold standard for accurate identification, crucial for guiding antifungal therapy and improving patient management.

Keywords: Head and neck cancer, Candidiasis, *Rhodotorula*, *Cyberlindnera fabianii*, Chromogenic media, PCR-RFLP, ITS sequencing, Pakistan

1. INTRODUCTION

Head and neck cancer (HNC) constitutes a significant public health burden in Pakistan, ranking among the most common malignancies (Qamar *et al.*, 2024), with a particularly high prevalence in Southeast Asia (Answer *et al.*, 2018). The primary treatment modalities—surgery, radiotherapy, and chemotherapy often compromise the oral mucosal barrier and local immunity, creating a favorable environment for opportunistic infections (Chavan *et al.*, 2023; Almarzooqi *et al.*, 2023; Barsouk *et al.*, 2023). Oral candidiasis is a frequent and debilitating complication in these patients, associated with increased morbidity, treatment interruptions, and diminished quality of life (Kermani *et al.*, 2021; Praksh *et al.*, 2023).

While *Candida albicans* has historically been the predominant agent, a global epidemiological shift towards non-*albicans* *Candida* (NAC) species and other non-*Candida* yeasts (NCY) is well-documented in immunocompromised populations, including cancer patients (Otto and Babady, 2023; Nguyen *et al.*, 2024). This shift concerns species such as *C. glabrata*, *C. tropicalis*, and *C. krusei* exhibit variable and often reduced susceptibility to common antifungal agents like fluconazole (Singh and Chakrabarti, 2017; Sharma and Chakrabarti, 2023). The epidemiology varies geographically; for instance, South Asia reports a significantly higher prevalence of invasive candidiasis compared to Western nations (Singh and Chakrabarti, 2017). Furthermore, the spectrum of fungal pathogens is broadening. Beyond *Candida*, infections caused by *Aspergillus*, *Mucor*, and *Cryptococcus* have increased in immunocompromised hosts (Gnat *et al.*, 2021). Emerging yeasts like *Rhodotorula* spp. (e.g., *R.*

mucilaginosa, *R. glutinis*) are now recognized as human pathogens, with invasive disease notably reported in Asia (Al-Ammari and Hussein, 2020; Miglietta *et al.*, 2015), and rare opportunistic yeasts pose a significant challenge in oncological settings (Chitasombat *et al.*, 2012). The recent identification of species like *Cyberlindnera fabianii* in clinical settings further underscores this evolving landscape (Fan *et al.*, 2023; Sharma and Chakrabarti, 2023). Accurate identification to the species level is, therefore, no longer an academic exercise but a clinical imperative for guiding appropriate antifungal therapy and improving patient care (Chitasombat *et al.*, 2012).

Conventional identification based on culture, assimilation, and fermentation tests is time-consuming, complex, and may lack the resolution to distinguish closely related species, posing challenges for routine laboratories (Freydiere *et al.*, 2009; Nadeem *et al.*, 2010). Chromogenic culture media have improved turnaround times for common *Candida* species by producing species-specific colony colors and are valued as easy, quick, and cost-effective tools (Hulimane *et al.*, 2018; Vecchion *et al.*, 2017). However, their spectrum is limited, often failing to identify uncommon or emerging yeast species (Jabroodini *et al.*, 2024). Molecular techniques, particularly PCR amplification of the highly variable Internal Transcribed Spacer (ITS) regions of ribosomal DNA, followed by restriction analysis (RFLP) or sequencing, offer a more sensitive, specific, and rapid alternative (Nagla *et al.*, 2018; Karimi *et al.*, 2015). Although PCR-RFLP is a useful rapid method, its utility can be constrained by the lack of restriction sites for certain enzymes in many yeast species, limiting differentiation (Kianipour *et al.*, 2018; Alagiri *et al.*, 2017).

In Pakistan, despite the high burden of HNC and the critical nature of fungal infections in immunocompromised patients, data on the precise etiology of oral yeast infections in HNC patients remains sparse and the area has been relatively neglected (Singh and Chakrabarti, 2017;). This study employs a multi-technique approach—from phenotypic screening on chromogenic media to molecular confirmation via PCR-RFLP and definitive ITS sequencing—to comprehensively profile the oral yeast flora in a cohort of Pakistani HNC patients. We compare the efficacy of these diagnostic methods and report on the isolation of clinically significant yeast species, including the first documented isolation of *Cyberlindnera fabianii* from an HNC patient in Pakistan.

2. MATERIALS AND METHODS

2.1. Study Design, Ethics, and Sample Collection

This cross-sectional study was approved by the Institutional Review Board of BUIITEMS (Ref. FLS&I/BUIITEMS/5398). After obtaining informed consent, 110 non-repeated saliva samples were collected from histo-pathologically confirmed HNC patients at the Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN) Hospital between December 2018 and April 2019. Sampling was irrespective of age, gender, or treatment status (naive, on chemotherapy, radiotherapy, or chemoradiotherapy). A control group of 100 saliva samples was collected from healthy volunteers with no history of cancer or chronic immunosuppression.

2.2. Phenotypic Isolation and Identification

Samples were inoculated onto Yeast Extract Malt Extract (YM) agar and incubated at 37°C for 48-96 hours. Pure yeast colonies were obtained by sub-culturing. Preliminary phenotypic identification was performed on two chromogenic media: Brilliance™ *Candida* Agar (BCA, Oxoid) and CHROMagar™ *Candida* Plus (CCP). Plates were incubated at 35°C for 48 hours, and species were presumptively identified based on colony color and morphology as per manufacturers' guidelines.

2.3. Molecular Characterization

DNA Extraction and PCR: Genomic DNA was extracted from pure yeast cultures using a modified CTAB method (Miglietta *et al.*, 2015). The ITS region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Chitasombat *et al.*, 2012). The PCR profile included initial denaturation at 95°C for 6 min, followed by 35 cycles of 95°C for 40 s, 59°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 7 min.

PCR-RFLP: Approximately 10 µL of purified PCR product was digested with the *MspI* restriction enzyme in a 32 µL reaction volume at 37°C for 3 hours. Digested fragments were separated on a 2% agarose gel and visualized.

DNA Sequencing and Phylogenetic Analysis: Representative PCR amplicons for each distinct banding pattern or chromogenic phenotype were purified and sequenced bidirectionally using the Sanger method. The obtained sequences were curated and aligned using MEGA6 software (Fan *et al.*, 2023). They were compared to reference sequences in the NCBI GenBank database using BLASTn. A phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replications to confirm species identity and illustrate genetic relationships.

2.4. Statistical Analysis

Data was analyzed using IBM SPSS Statistics version 22. Descriptive statistics summarized demographic and clinical variables. Binary logistic regression was used to assess the association between participant type (HNC patient vs. healthy control) and yeast infection status. Multinomial logistic regression examined the relationship between the dependent variable (identified yeast species) and independent variables (age, gender, treatment type). A mixed model analysis was employed to handle cases with multiple yeast species isolated from a single sample. A p -value of <0.05 was considered statistically significant.

3. RESULTS

3.1. Participant Demographics

The study enrolled 110 HNC patients (91.8% male, 8.2% female) with a mean age of 49.2 years, and 100 healthy controls (88% male, 12% female). Among patients, 40% were undergoing concurrent chemoradiotherapy, 20.9% chemotherapy alone, 14.5% radiotherapy alone, and 24.5% were untreated at sampling stage (Fig.1, Table 1).

Table 1. Sociodemographic and clinical characteristics.	N (110)	%
Gender		
Male	101	91.8
Female	09	8.1
Age groups		
20-29	03	3
30-39	28	25
40-49	38	35
50-59	22	20
60-69	18	16
70-79	1	1
Treatments		
chemoradiotherapy	44	40
chemotherapy	23	20.9
Radiotherapy	16	14.5
Not treated	27	24.5
Healthy participants		
Male	88	88
Females	12	12

3.2. Yeast Isolation and Phenotypic Identification

Culture on YM agar yielded 126 yeast isolates from 110 HNC patients (some patients had mixed infections) and 31 isolates from 100 healthy controls. On chromogenic media, CCP successfully differentiated six *Candida* species (*C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. lusitaniae*), while BCA could only reliably differentiate four, failing to separate *C. albicans* from *C. dubliniensis* and *C. glabrata* from *C. lusitaniae* (Table 2).

3.3. Molecular Identification and Diversity

PCR amplification of the ITS region produced bands ranging from approximately 377 bp to 871 bp. DNA sequencing of these amplicons revealed a total of 11 distinct yeast species from the HNC patient cohort (Fig. 2). The most prevalent species were *Rhodotorula mucilaginosa* (29 isolates) and *Candida tropicalis* (26 isolates). Notably, we identified less common species, including *Candida gattii* (2 isolates) and *Cyberlindnera fabianii* (1 isolate). In contrast, only three species (*C. albicans*, *C. glabrata*, *R. mucilaginosa*) were found in the healthy control group.

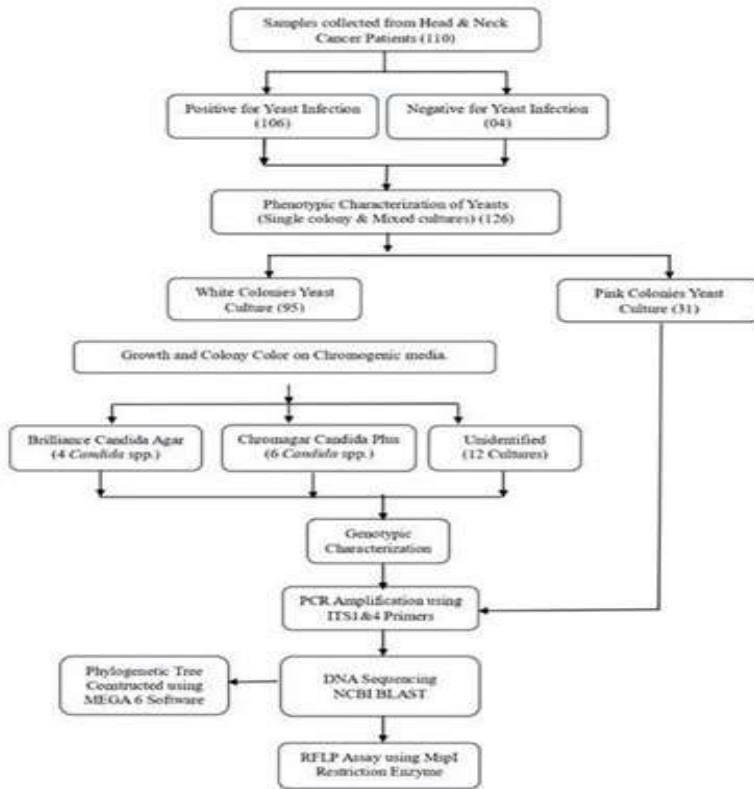








Fig. 1. The study utilized flow charts to isolate human pathogenic yeast strains from head and neck cancer patients.

Table 2. Colony color variations of *Candida* species on two different chromogenic media.

<i>Candida</i> species	Brilliance <i>Candida</i> agar (BCA)		CHROMagar <i>Candida</i> Plus (CCP)	
	Colony	Color	Colony	Color
<i>C. tropicalis</i>		Metallic Blue (26/0)		Dull Blue with pink background (26/0)
<i>C. glabrata</i>		Beige (14/2)		Mauve (11/2)
<i>C. albicans</i>		Dark Sea Green (39/27)		White Colonies with blue halo (25/27)











<i>C. parapsilosis</i>		Rust Brown (4/0)		White rough colonies with light blue background (4/0)
<i>C. dubliniensis</i>		Dark Sea Green (0/0)		Sea Green (14/0)
<i>C. lusitaniae</i>		Beige (0/0)		White, Purple (3/0)
Unidentified		Purplish Grey (6/0)		Purple Sea green (6/0)
Unidentified		White (6/0)		White (6/0)
Total		(95/29)		(95/29)



Fig. 2. Gel image of PCR-amplified products of the ITS regions of *C. fabianii* (1), *C. kefyr* (2), *C. dubliniensis* (3), *C. guilliermondii* (4), *C. gattii* (5), *C. glabrata* (6), the DNA ladder (100 bp), *C. parapsilosis* (7), *R. mucilaginosa* (8), *C. tropicalis* (9), *C. lusitaniae* (10), *C. albicans* (11) and the negative control (c).

3.4 Molecular Phylogeny

Fungus specific universal primer pair (ITS1 and ITS4) were able to successfully amplify the ITS region of all (126) yeast tested, providing a PCR product of approximately 377 -871 bp (Fig. 2). Representative samples from each distinct PCR product size were sequenced using Sanger sequencing. The results of the BLAST search and phylogenetic analysis show a significant level of genetic similarity among yeast species. Several species of the yeast species demonstrated 99% homology, including *Candida albicans* (25), *C. tropicalis* (26), *C. dubliniensis* (14), *C. gattii* (2), *C. glabrata* (11), and *C. parapsilosis* (4). Furthermore, *C. lusitaniae* had a 98% similarity, while *R. mucilaginosa* had 91% homology. Other species, such as *C. fabianii*, demonstrated 83% homology, *C. guilliermondii*, 52% homology, and *C. kefyr*, had a lower similarity of 28%. These findings showed that closely related species may have significant genetic similarities (Fig. 3).

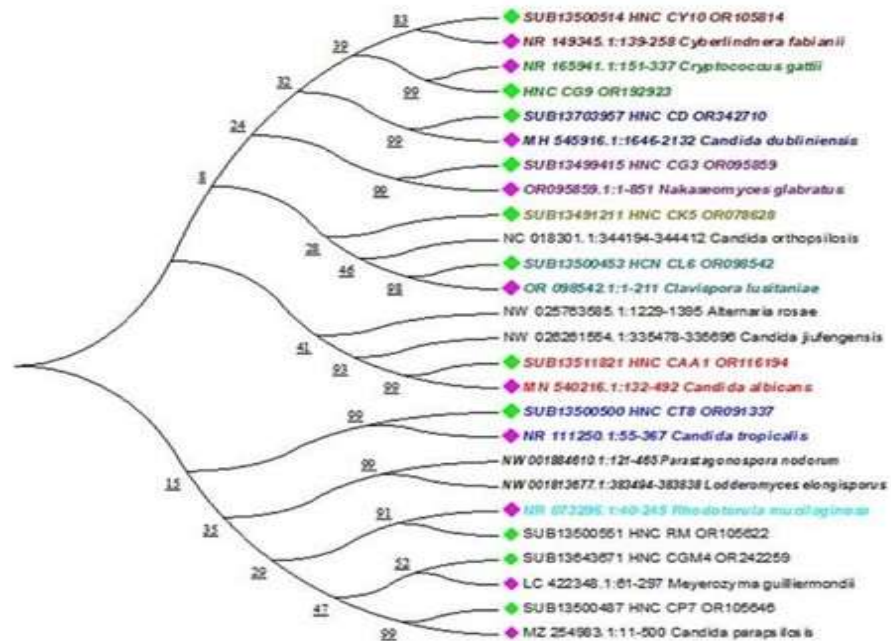


Fig. 3. Phylogenetic tree comprising eleven identified yeast species. The green bullets indicate our sequences, as opposed to the pink bullets of reference sequences.

Restriction Fragment Length Polymorphism (RFLP) Analysis

PCR amplicons of ITS region were digested with *MspI* restriction enzyme, as described in material and methods. The products of digestion are shown in Table 3 and Fig 4, showing that the bands generated corresponded to the predicted size. The digestion of ITS regions of yeast species by *MspI* generate two bands (239, 298) for of *C. albicans*, *C. dubliniensis* (239, 298), *C. glabrata* (320, 561), *C. lusitanae* (118, 264), *C. tropicalis* (186, 340) and three bands (82, 155, 370) for *C. guilliermondii*. However, there were no restriction sites for *MspI* in the ITS region of *C. parapsilosis*, *C. kefyrr*, *C. fabianii*, *C. gattii*, *R. Mucilaginosa*. However, the PCR and digestion products for the above species were of the same sizes. Notably, the restriction patterns of *C. dubliniensis* and *C. albicans* were identical, making it difficult to distinguish between them using the RFLP assay.

Table 3. Size of ITS 1 and 4 regions before and after endonuclease digestion with *MspI* enzyme.

No.	Yeast species	Size of ITS 1 and 4	Size of <i>MspI</i> -RFLP Fragment	Accession No
01	<i>C. albicans</i>	535bp	239, 298	OR116194
02	<i>C. dubliniensis</i>	540bp	239, 298	OR342710
03	<i>C. glabrata</i>	871bp	320, 561	OR095859
04	<i>C. guilliermondii</i>	610bp	82, 155, 370	OR242259
05	<i>C. kefyrr</i>	720bp	720	OR098542
06	<i>C. lusitanae</i>	377bp	118, 264	OR078628
07	<i>C. parapsilosis</i>	520bp	520	OR105646
08	<i>C. tropicalis</i>	524bp	186, 340	OR091337
09	<i>C. gattii</i>	600bp	600	OR192923
10	<i>C. fabianii</i>	629bp	630	OR105814
11	<i>R. mucilaginosa</i>	630bp	630	OR105622

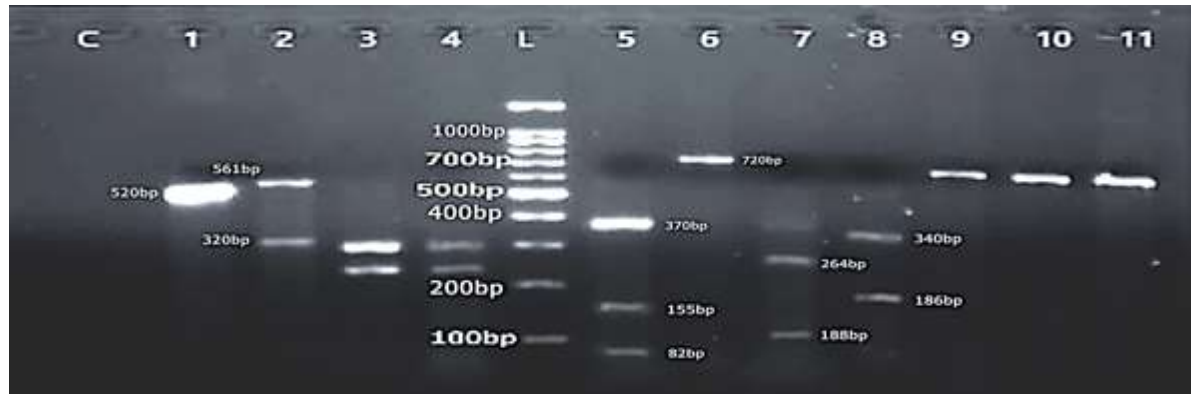


Fig. 4. Gel image of the PCR–RFLP assay: control (C) *Candida parapsilosis* (1), *C. glabrata* (2), *C. dubliniensis* (3), *C. albicans* (4), DNA ladder (100 bp), *C. guilliermondii* (5), *C. kefir* (6), *C. lusitaniae* (7), *C. tropicalis* (8), *C. fabianii* (9), *C. gattii* and *R. mucilaginosa* (11).

Yeast Distribution Among Cancer Patients and Healthy Participants

Results showed that HNC patients demonstrated a significantly higher incidence of yeast infections compared to healthy participants (Table 4). Eleven distinct yeast species were identified in HNC patients, while only three species were found in the healthy group. The most common species in healthy participants were *C. albicans* (27), with two samples of *C. glabrata* and two samples of *R. mucilaginosa* whereas the most prevalent yeast species in HNC patients were *R. mucilaginosa* (29), followed by *C. tropicalis* (26), while less common yeast species were *C. fabianii* (1), *C. gattii* (2), and *C. lusitaniae* (3).

Table 4. Distribution of yeast species in HNC patients as compared to healthy participants.

No.	Yeast species	Cancer patients	Healthy Participants
1	<i>Candida albicans</i>	25	27
2	<i>C. dubliniensis</i>	14	0
3	<i>C. glabrata</i>	11	2
4	<i>C. guilliermondii</i>	5	0
5	<i>C. kefir</i>	6	0
6	<i>C. lusitaniae</i>	3	0
7	<i>C. parapsilosis</i>	4	0
8	<i>C. tropicalis</i>	26	0
9	<i>C. gattii</i>	2	0
10	<i>C. fabianii</i>	1	0
11	<i>R. mucilaginosa</i>	29	2
	Total	126	31

Gender-specific Distribution of Yeast Species

The distribution of yeast species varied between male and female cancer patients. In male HNC patients, eleven yeast species were identified, whereas seven yeast species were detected in female cancer patients. In males, the most prevalent species were *R. mucilaginosa* (28), *C. tropicalis* (24), and *C. albicans* (24), whereas *C. fabianii* (1), *C. gattii* (1), and *C. lusitaniae* (2) were less common. The most prevalent yeast species found in female cancer patients were *C. glabrata* (2) and *C. tropicalis* (2). Overall, the incidence of yeast infections was greater in male patients (Table 5).

Table 5. Gender-wise distribution of finally identified yeast species.

No.	Yeast species	Gender		
		Male	Female	Total
1.	<i>Candida albicans</i>	24	1	25
2.	<i>C. dubliniensis</i>	14	0	14
3.	<i>C. glabrata</i>	9	2	11
4.	<i>C. guilliermondii</i>	5	0	5
5.	<i>C. kefyri</i>	6	0	6
6.	<i>C. lusitaniae</i>	2	1	3
7.	<i>C. parapsilosis</i>	3	1	4
8.	<i>C. tropicalis</i>	24	2	26
9.	<i>C. gattii</i>	1	1	2
10.	<i>C. fabianii</i>	1	0	1
11.	<i>R. mucilaginosa</i>	28	1	29
Total		117	9	126

Age-specific Distribution of Yeast Infections

The 40-49- and 50-59-year-old age groups had the highest rate of yeast infections, with 10 yeast species identified, followed by the 30-39 age group with nine yeast species. The most prevalent yeast species in the 40-49 and 30-39 age groups were *C. tropicalis*, *R. mucilaginosa*, and *C. albicans*, respectively, while *R. mucilaginosa* and *C. albicans* were the most common yeast species in the 50-59 age group. Seven yeast species were detected in the 60-69 age range, but only one yeast species *C. tropicalis* was identified in the 70-79 age group (Table 6).

Table 6. Frequency distribution of yeast species in various age groups of cancer patients.

No.	Yeast species	Age groups						Total
		20-29	30-39	40-49	50-59	60-69	70-79	
1	<i>Candida albicans</i>	0	8	8	7	2	0	25
2	<i>C. dubliniensis</i>	1	2	6	3	2	0	14
3	<i>C. glabrata</i>	0	2	4	2	3	0	11
4	<i>C. guilliermondii</i>	0	1	2	2	0	0	5
5	<i>C. kefyri</i>	0	3	1	1	1	0	6
6	<i>C. lusitaniae</i>	0	0	1	2	0	0	3
7	<i>C. parapsilosis</i>	0	1	1	1	1	0	4
8	<i>C. tropicalis</i>	0	9	11	3	2	1	26
9	<i>C. gattii</i>	0	0	1	1	0	0	2
10	<i>C. fabianii</i>	0	1	0	0	0	0	1
11	<i>R. mucilaginosa</i>	2	6	10	6	5	0	29
Total		3	33	45	28	16	1	126

Treatment-wise Distribution of Yeast Species

In this study, HNC patients undergoing chemo-radiotherapy had a higher incidence of yeast infections as compared to those receiving chemotherapy or radiation therapy alone. Ten yeast species have been identified in chemoradiotherapy patients; the most prevalent species were *C. tropicalis* (15) and *R. mucilaginosa* (11), with the rarest species, *C. fabianii*, finding in patients receiving both radiation and chemotherapy. Eight yeast species were found in individuals receiving just chemotherapy, in which the most common species were *C. dubliniensis* (5), *C. tropicalis* (5), and *R. mucilaginosa* (5). On the other hand, seven yeast species were found in patients receiving radiation alone and in individuals whose treatment had not yet started. The most common yeast species among patients undergoing radiation therapy were *C. glabrata* and *R. mucilaginosa*, while the most common yeast species among those who were not treated were *C. albicans*, which is often found in the oral microbiome (Table 7).

Table 7. Frequency distribution of yeast species taking different treatments.

No.	Yeast species	Treatment Type				Total
		Chemo-Radiotherapy	Chemotherapy	Radiotherapy	Not treated	
1	<i>Candida albicans</i>	6	2	1	16	25
2	<i>C. dubliniensis</i>	5	5	1	3	14
3	<i>C. glabrata</i>	4	1	4	2	11
4	<i>C. guilliermondii</i>	4	1	0	0	5
5	<i>C. kefyr</i>	2	2	0	2	6
6	<i>C. lusitaniae</i>	2	0	1	0	3
7	<i>C. parapsilosis</i>	0	1	3	0	4
8	<i>C. tropicalis</i>	15	5	3	3	26
9	<i>C. gattii</i>	1	0	0	1	2
10	<i>C. fabianii</i>	1	0	0	0	1
11	<i>R. mucilaginosa</i>	11	5	4	9	29
	Total	55	22	18	37	126

Binary Regression Analysis:

Logistic regression (LR) analysis with enter likelihood ratio method model was performed to examine the influence of various risk factors (independent variables) i.e., participant type (cancer patients and healthy participants) on the association of yeast infection (dependent variable) to predict the value of infection. The Omnibus Tests of Model Coefficient during LR analysis shows significant value of Chi-square (113.117, $p=0.00$ $n=420$). The variables in the equation for participant type indicating significant ($p<0.05$) value with odds ratio (OR) 1.877, 95% confidence level (CI) 19.940-174.476 (Table 8). The overall regression model was statistically significant with the value of Nagelkerke $R^2=0.574$ indicating that 57.4% yeast infection (+ve or -ve) is explained by type of participants i.e. cancer patients and healthy individual (Table 9).

Table 8. Output of variables in equation of Binary Logistic Regression

Step 1a: Independent Variables	B	S.E.	Wald	df	Sig.	Exp(B)	95% CL for Exp (B)	
							Lower	Upper
Participants	0.630	0.145	18.873	1	0.000	1.877	19.940	174.476

Table 9. Model summaries of the binary logistic regression

Step	-2 Log likelihood	Cox and Snell R Square	Nagelkerke R Square
1	158.186 ^a	0.416	0.574

a. Estimation terminated at iteration number 6 because parameter estimates changed by less than 0.001.

Multinomial Logistic Regression Analysis:

The multinomial logistic regression was conducted to examine the relationship between yeast infection (dependent variable) the outcome of which is more than two categories and various independent variables (Table 10). The dependent variable consisted of 11 different yeast species which were identified through PCR amplification of ITS regions. Whereas the independent variables included gender, age group and treatments. Negative value was selected as a reference category.

The model fitting information of multinomial Logistic Regression clearly indicated that it is appreciably fit and explained the association between yeast infection (dependent variable) and all independent variables (χ^2 232.523, df 110, significance $p < 0.000$). The Goodness of Fit model also verified that the model is highly acceptable since the Pearson value of Chi square (143.654, df 319) is highly significant (1.000 i.e. > 0.05). The calculated value of R^2 Cox and Snell and Nagelkerke was 0.448 clearly indicated that the yeast infection is significantly (48.9%) explained by the respondent (independent) variables. The model also predicted the outcomes (χ^2 (11) = 56.608, $p = 0.00$) and (χ^2 (33) = 49.143, $p < 0.035$), explaining significant relations of yeast infection (dependent variable) with that type of participants (patients vs. healthy) and treatments, respectively (Table 10).

Table 10. Likelihood Ratio Tests of Multinomial logistic regression.

Effects (Independent Variables)	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood of Reduced Model	X^2	df	Sig.
Intercept	332.234 ^a	0.000	0	0.000
Patients vs. Healthy	388.842 ^b	56.608	11	0.000
Gender	347.226 ^b	14.993	11	0.183
Treatments	381.376 ^b	49.143	33	0.035
Age Groups	381.219	48.985	55	0.703

The chi-square statistic is the difference in -2 log-likelihoods between the final model and a reduced model. The reduced model is formed by omitting an effect from the final model. The null hypothesis is that all parameters of that effect are 0.

- This reduced model is equivalent to the final model because omitting the effect does not increase the degrees of freedom.
- Unexpected singularities in the Hessian matrix are encountered. This indicates that either some predictor variables should be excluded, or some categories should be merged.

Mixed Model Analysis

As a result of culture test and application of various diagnostic techniques, it was observed that several samples had mixed yeast infection *i.e.*, more than two (2) yeast species were encountered from a single sample. Therefore, a mixed model analysis was conducted through making duplicate copies of samples, each indicting any one of the yeast species infection or negative. The multiple yeast species infection association was predicted in relation to patients' type (healthy or cancer), age group, gender and treatment. It was observed that all the variables under study showed significant relations with the occurrence of yeast infection in different interactions. The estimated effects of significant relations between various variables reflected in SPSS output file were determined and summarized in terms of estimates, standard error, degree of freedom (df), t value, significance (p value), 95% confidence interval (lower and upper boundaries) in Table 11.

Table 11. Summarized Estimates of Fixed Effects through Mixed Model Analysis.

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-12.044	5.469	380	-2.202	0.028	-22.798	-1.291
(Gender=1)	ocw6	5.312	380	2.268	0.024	1.600	22.488
(Treatment=2)	11.493	5.153	380	2.230	0.026	1.361	21.626
(Treatment=3)	10.493	4.046	380	2.594	0.010	2.539	18.448
(Age_Groups=2)	12.378	5.622	380	2.202	0.028	1.323	23.432
(Age_Groups=3)	12.378	5.622	380	2.202	0.028	1.323	23.432
(Age_Groups=4)	12.711	5.312	380	2.393	0.017	2.267	23.155
(Age_Groups=5)	12.044	5.622	380	2.142	0.033	0.990	23.099
(Gender=1) * (Treatment=2)	-10.093	5.079	380	-1.987	0.048	-20.079	-0.108
(Gender=1) * (Treatment=3)	-9.538	3.710	380	-2.571	0.011	-16.833	-2.243
(Gender=1) * (Age_Groups=2)	-11.423	5.490	380	-2.081	0.038	-22.218	-0.628
(Gender=1) * (Age_Groups=3)	-11.611	5.485	380	-2.117	0.035	-22.395	-0.827
(Gender=1) * (Age_Groups=4)	-12.593	5.120	380	-2.460	0.014	-22.661	-2.526
(Gender=1) * (Age_Groups=5)	-11.737	5.505	380	-2.132	0.034	-22.561	-0.913
(Treatment=2) * (Age_Groups=5)	-13.216	6.562	380	-2.014	0.045	-26.119	-0.312

4. DISCUSSION

This study provides a comprehensive molecular analysis of the oral yeast flora in Pakistani HNC patients, revealing a significantly more diverse ecosystem than previously appreciated through conventional means. Our finding of 11 different species underscores the complexity of oral mycobiome dysbiosis in this immunocompromised population and aligns with the global trend of expanding fungal pathogen diversity (Gnat *et al.*, 2021; Sharma and Chakrabarti, 2023).

The demographic profile of our cohort, with a marked male predominance (91.8%), corresponds with the established epidemiology of HNC in Pakistan and Southeast Asia, where risk factors like tobacco and betel nut use are more common among males (Answer *et al.*, 2018; Hashim *et al.*, 2019). The superior differentiation capability of CHROMagar™ Candida Plus (CCP) over Brilliance Candida Agar (BCA) aligns with recent evaluations of chromogenic media (Tamura *et al.*, 2022). CCP successfully differentiated six *Candida* species, consistent with its design for presumptive identification (Soni *et al.*, 2017), while BCA's performance was more limited, as observed in other comparative studies (Vecchioni *et al.*, 2017). However, the failure of both media to identify non-*Candida* yeasts like *R. mucilaginosa* and the rare *C. fabianii* highlights a critical limitation: these media are designed primarily for common *Candida* spp. and should be used only as screening tools in high-risk populations where uncommon yeasts may be present (Jabrodoni *et al.*, 2024; Ganguli *et al.*, 2024).

The PCR-RFLP method, while cost-effective and rapid, also showed limitations that echo previous findings. The digestion patterns we observed for species like *C. guilliermondii* corroborate earlier work (Jafari *et al.*, 2017; Habib *et al.*, 2016). However, the identical RFLP profile for *C. albicans* and *C. dubliniensis* using *MspI* is a known drawback, necessitating additional methods for differentiation (Kianipour *et al.*, 2018; Allam and Salem, 2012). Furthermore, the lack of a restriction site for *MspI* in species such as *C. parapsilosis* and *C. gattii* (Alagiri *et al.*, 2017), which constituted a notable portion of our isolates, renders this specific protocol inadequate for standalone diagnosis in our setting.

Therefore, DNA sequencing of the ITS region proved indispensable as the definitive method, confirming its utility as reported elsewhere (Karimiet *et al.*, 2015; Hemaïd *et al.*, 2021). It not only resolved ambiguities but also led to the identification of species of major clinical importance. The predominance of *Rhodotorula mucilaginosa*, a yeast known for intrinsic resistance to fluconazole and echinocandins, is a finding of particular concern and consistent with its emerging role as an opportunistic pathogen in Asia (Al-Ammari and Hussein, 2020; Miglietta *et al.*, 2015). The isolation of *Cyberlindnera fabianii* (syn. *Candida fabianii*), previously reported in nosocomial outbreaks and fungemia, marks its first documentation from an HNC patient in Pakistan and adds to the global profile of this emerging pathogen (Fan *et al.*, 2023; Sharma and Chakrabarti, 2023). The strong statistical association between HNC patient status and yeast colonization/diversity reinforces the concept of cancer therapy-induced immunosuppression and mucosal damage as key risk factors, a phenomenon well-documented in the literature (Kermani *et al.*, 2021; Prakash *et al.*, 2023).

4.1. Clinical and Public Health Implications

- **Diagnostic Strategy:** In resource-limited settings like Pakistan, a tiered approach is recommended. Chromogenic media can be used for initial, rapid screening. However, for symptomatic infections or treatment failure, samples should be referred for molecular identification, ideally via ITS sequencing, to guide appropriate therapy.
- **Antifungal Stewardship:** The high prevalence of non-albicans species and azole-resistant yeasts like *R. mucilaginosa* suggests that empirical use of fluconazole may be suboptimal in this patient group. Antifungal susceptibility testing should be encouraged where feasible.
- **Epidemiological Surveillance:** The presence of emerging and rare yeasts calls for continuous surveillance to monitor epidemiological shifts and prevent potential outbreaks in oncology wards.
- **Statistical association:** The strong statistical association between HNC patient status and yeast colonization/ diversity reinforces the concept of cancer therapy-induced immunosuppression and mucosal damage as key risk factors.

5. Conclusion

This study demonstrates a rich diversity of oral yeast species, including emerging and uncommon pathogens, in Pakistani HNC patients. While phenotypic methods are useful for initial screening, they significantly underestimate mycological diversity. PCR-RFLP with a single enzyme has limited utility due to a lack of universal restriction sites. DNA sequencing of the ITS region remains the most accurate method for species-level identification, which is critical for implementing effective antifungal strategies and improving outcomes in this vulnerable population. The finding of *Cyberlindnera fabianii* warrants further attention to its clinical significance and transmission dynamics in healthcare settings.

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