American Journal of Biology and Natural Sciences
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Fungal Contamination of Restaurant Cold Foods in Kirkuk, Iraq: Bena (B-Tubulin)–Based Mold Identification and PLB1 Screening in Candida Isolates

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Received: 2025, 15, Dec
Accepted: 2026, 19, Jan
Published: 2026, 21, Feb

Abstract: Background: Fungal spoilage of restaurant foods, especially ready-to-eat and minimally processed products, is a pervasive issue due to the ability of yeasts and molds to withstand preparation and holding steps as well as impact upon shelf life with potential for consequences to hygiene control. RTE matrix like salads are highly susceptible to carry over and post-processing contaminations and surveys have been reported to have elevated yeast–mold counts beyond the permissible limits. Airborne dissemination in busy food-service settings may also play a role in the contamination of surfaces and exposed foods, therefore confirming the requirement for species-level identification. Thus, we examine the occurrence of mycological contamination in foods from restaurants employing conventional (culture-based) methods and validate mold identification with BenA (β-tubulin) sequences while assessing yeast isolates for virulence-associated PLB1 gene to contribute with a risk-aware interpretation.

Materials and Methods: A total of 93

sinonasal swabs were taken from restaurant workers in Kirkuk, Iraq during the period from August to September 2025. Fungal strains were identified by culture morphology from SDA and CHROMagar Candida and then observed under a microscope. Phenotypic phospholipase activity was determined on egg yolk agar and molecular identification by PCR targeting β-tubulin and PLB1 genes.

Results: Fungal growth was detected in 79.17% of samples, with Candida species predominating, particularly Candida krusei. Phenotypic phospholipase activity was absent among the yeast isolates. Molecular analysis confirmed accurate species-level identification of molds and revealed that 81.58%% of Candida isolates carried the PLB1 gene.

Conclusions: restaurant cold foods exhibited significant yeast-dominated fungal contamination (Candida krusei in particular) and occasional molecularly proven molds - chances are for enzymes that save toxins but all assay negative.Furthermore, the PLB1 carriage despite 100% phenotypic phospholipase negativity supports colonization-driven condition-dependent virulence expression which due to resistant biofilms may require tighter hygiene controls.

Keywords: Molecular diagnosis, Candida spp, Molds, virulence factors, BenA, PLB1

Introduction

Restaurant foods, ready-to-eat (RTE) and minimally processed in particular, are commonly and time again involved in food safety / quality issues as yeasts and molds may prevail along preparation, holding and serving steps; lower a product shelf life when participating to spoilage; point out weaknesses in hygiene controls. In a microbiological evaluation of ready-to-eat (RTE) cooked foods, for example, the prevalence of yeasts and moulds above reference standards in RTE foods was 78.4 % but concerns about 14.4 % such products were based on microbial criteria as unsatisfactory samples -indicating how often fungal indicators may exceed acceptable cutoff levels within actual food-service systems [1].

Fungal contamination of RTE-products is particularly in the spotlight in moist-nutritious products like salads as it appears that washing R June 2018 Data Sheet: Foodborne disease, outbreaks and recalls/Foodborne Pathogens & Disease 613-618fresh # Mary Ann Liebert Inc /Vol. A survey of commercial RTE salads showed the prevalence levels of yeast–mold ranging from 1.00 to 7.00 log CFU/g in most of the tested products, which indicates a wide range and occasionally high contamination potential [2]. In a second study of both restaurant and street-vended vegetable salads, the fungal load was found to be approximately 3.96–5.06 log10 CFU/g and above the national standards in all samples, suggesting that ready-to-eat (RTE) foods sold at food-service establishments may often have significant levels of fungi [3].

In addition to direct contact with hands, the work environment in a restaurant could also potentially cause airborne spread and deposition. Bioaerosol monitoring in food-court environments has indicated that fungi can be a major component of culturable airborne microorganisms, also suggesting the potential for deposition onto food-contact surfaces and exposed foods during peak cooking or serving times [4]. These facts underline the importance of correct identification of food-associated molds for risk assessment, as well as for control approach destination.

While colony characters continue to be an important screening tool, there are limitations resulting from phenotypic overlap that reduce the use of colonial characteristics for species-level identification in some major food-related lineages such as Eurotiales. Modern Eurotiales systematics are based on a gene approach enabling the clear delimitation of closely related genera and species [5]. In this regard, the β-tubulin gene region (BenA/BT2) is among the most informative marker for Eurotiales molds and has been adopted in updated identification systems and taxonomic databases [6,5]. In addition to identifying mold, virulence-related potential in yeast isolates can reinforce interpretation in the context of risk; traits associated with phospholipase are some of the most well-described mechanisms of pathogenicity and there is experimental evidence that PLB1 contributes directly to in vivo pathogenicity rendering it a biologically relevant molecular marker, complementary to phenotypic testing [7]. In this context, the combination of surveillance in restaurant foods for fungal contamination along with BenA-based mold identification and PLB1 as yeast virulence marker could develop a strong platform in line with the increasing concerns at worldwide level on disease causing fungi preeminentled by WHO campaign on fungal priority pathogens [8].

In light of these considerations, the present study aims to isolate and characterize fungi from restaurant food samples using conventional culture-based methods, to improve species-level identification of mold isolates by targeting the *BenA* (β-tubulin) gene, and to screen yeast isolates for the virulence-associated *PLB1* gene as a molecular indicator of phospholipase-related pathogenic potential.

Materials and methods

Sample collection: Forty-eight cold-food samples were collected during the period from August 2025 to September 2025. The sampled foods comprised three types of cold dishes: hummus with tahini, tuffahiya, and tzatziki. Sixteen samples of each food type were obtained from 16 different

restaurants across several areas of Kirkuk city. The restaurants were categorized into two groups: high-end restaurants (n = 8), from which eight samples of each cold dish were collected, and popular restaurants (n = 8), from which eight samples of each cold dish were also collected. Samples were placed in dedicated plastic containers and stored at 4°C for subsequent analysis.

In accordance with ISO (International Organization for Standardization) recommendations, 25 g of each sample were homogenized with 225 mL of peptone broth [9]. The suspension was set at room temperature for 2–4 h to ensure that the food sample distributed well in broth, and it was thoroughly shaken. Three other serial dilutions were attainted by inoculating 1 mL traversed through to the test tubes with 9 mL of peptone broth. Aseptically, fungi was isolated using the pour-plate technique by pipetting 100 µL from the first and third dilutions into sterile Petri dishes and adding not more than 15 ml of sterile Sabouraud Dextrose Agar (SDA) and mixed gently to ensure homogeneity. After solidification, the plates were incubated at 25°C for 5 days.

Isolation of fungi: The phenotypic characteristics of yeast colonies growing on SDA are identified by observing colony color, shape, texture, and elevation. This is followed by microscopic examination through preparing a glass slide from the colonies grown on the medium, where a small portion of the colony is taken using a sterile inoculating loop and placed on the slide, then stained with lactophenol cotton blue and covered with a coverslip. After preparation, the slide is examined under a light microscope at 40× magnification to observe the morphology of *Candida* cells and the presence of budding [10]. For molds, the phenotypic characteristics of colonies growing on SDA are identified by observing the colony color from both the obverse and reverse sides, as well as colony shape, texture, and elevation. Microscopic examination is then performed by preparing a slide from colonies grown on the medium, where a portion of the colony is collected using sterile adhesive tape and placed onto a slide that has been pre-coated with lactophenol cotton blue, then covered with a coverslip. After preparation, the slide is examined under a light microscope at 40× and 100x magnification to observe hyphal structures and conidia [11].

Identification

Chrome Candida Differential Agar (CCA):

Candida species were differentiated using a chromogenic agar medium, in which colony colors develop as a result of the interaction between the isolates and the chromogenic substrates in the medium[12]. After 24 h of growth on SDA, a small portion of each *Candida* colony was aseptically picked using a sterile inoculating loop and streaked onto CCA plates. The plates were then incubated at 37 °C for 24–48 h, resulting in the formation of colonies with distinct characteristic colors.

Phospholipase Activity Assay

Phospholipase activity of *Candida* strains was assessed on egg yolk agar as previously described. The basal medium was prepared by dissolving 65 g SDA, 58.4 g NaCl, and 5.5 g $CaCl_2$ in 980 mL of distilled water. Sterile egg yolk was centrifuged (5000 × g, 30 min, room temperature), and 20 mL of the resulting supernatant was incorporated into the cooled basal medium. Yeast suspensions were adjusted to 10^8 cells/mL, and 10 µL aliquots were spot-inoculated onto the plates, which were then incubated at 37 °C for 7 days. Enzymatic hydrolysis of lipid substrates in the medium results in the liberation of fatty acids, producing a precipitation zone around the colonies. Phospholipase activity was quantified by calculating the phospholipase index (Pz) according to Eq. (2), and classified as follows: negative (Pz = 1), weak (0.90–0.99), moderate (0.80–0.89), strong (0.70–0.79), and very strong (< 0.70) [13].

DNA Extraction and Molecular Identification

Fungal genomic DNA was extracted from a single pure colony using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Molecular

identification was carried out through amplification of the β-tubulin (*BT2*) gene using the primer pair Bt2a/Bt2b, as well as the phospholipase B1 (*PLB1*) gene using its specific primers[14,15]. The PCR products were verified by agarose gel electrophoresis. The amplified β-tubulin fragments were purified and sequenced (Macrogen Inc., Seoul, Korea), whereas the *PLB1* amplicons were evaluated only based on their expected band size and were not purified or sequenced. The obtained β-tubulin sequences were edited and trimmed using MEGA software (version 6), and subsequently analyzed by BLAST (NCBI database) to determine the closest related reference sequences [15].

Results and discussion

Identification

Laboratory culture of cold food samples collected from restaurants demonstrated a fungal isolation rate of 79.17%, with 38 positive samples out of 48 examined. From these positive samples, a total of 41 fungal isolates were recovered, indicating mixed fungal growth (two fungal species) in three samples. Yeasts predominated, with *Candida krusei* representing 26 isolates (63.41%), followed by *Candida glabrata* 7 isolates (17.07%), *Candida tropicalis* 3 isolates (7.32%), and *Candida parapsilosis* 2 isolates (4.88%). In contrast, molds were detected at low frequencies, with one isolate (2.44%) each of *Penicillium chrysogenum*, *Penicillium citrinum*, and *Aspergillus fumigatus*. Overall, these findings indicate a clear predominance of *Candida* isolates in the examined food samples, whereas filamentous fungi were recovered only sporadically.

The predominance of yeasts in restaurant cold-food samples is biologically plausible, as the literature indicates that *Candida spp.* can occur in diverse food matrices (particularly nutrient-rich and fermented foods) and that their presence raises food-safety concerns regarding food as a potential reservoir of opportunistic strains [16]. Crucially, with recent examination of ready-to-eat foods associating Candida contamination with food handling and storage such as hygienic practices of food handlers [17], this also supports the potential for human-to-fomite transfer within food preparation establishments where there is repeated manual contact. Additionally, contemporary genomic evidence reveals genetic affiliation/association between foodborne and clinical Candida isolates among common genogroups, indicative of shared reservoirs and potential bidirectional transmission under a One Health approach—emphasizing the importance to include analyses on both food and clinical isolates in the interpretation of contamination pathways [18].

Table (1): Distribution of Fungal Isolates Recovered from Sinonasal Swabs of Restaurant Workers

Fungal isolates	No. of Positive samples	Percentage
Candida krusei	26	63.41%
Candida glabrata	7	17.07%
Candida parapsilosis	2	4.88%
Candida tropicalis	3	7.32%
Penicillium chrysogenum	1	2.44%
Penicillium citrinum	1	2.44%
Aspergillus fumigatus	1	2.44%
Total	41	100%

The fungal isolates exhibited unique macroscopic and microscopic characteristics, which facilitated their morphological identification at genus and species levels. Candida isolates grew on Sabouraud Dextrose Agar (SDA) as creamy white to off-white, smooth, glistening colonies with a creamy consistency and convex profile generally with regular margins, whereas on the differential medium CHROMagar Candida Candida krusei formed highly characteristic rough spreading colonies having pale-pink centers and conspicuous white edge while Candida glabrata usually produced mauve pinkish purple colonies of more homogenous texture; Candida parapsilosis

showed pale (white/ light pink also creamy) colored colonies. Microscopically, Candida isolates dislayed ovoid to spherical yeast cells by budding singly and in small clusters as described for health friendly yeasts per (Figures 1,2) [19]. In contrast, *Penicillium* isolates exhibited fast-growing, velvety to powdery green colonies (often with a paler margin), and microscopy revealed the diagnostic “brush-like” (penicillus) conidiophores with phialides producing basipetal chains of single-celled conidia as shown in Figures (3,4)[20]. Finally, *Aspergillus fumigatus* exhibited a gray colony with white, floccose margins and a flat, smooth powdery surface, with a cream-colored reverse, and microscopic features consistent with the species, including hyaline septate hyphae and short conidiophores terminating in triangular-to-pyriform vesicles bearing a single layer of phialides that produced globose conidia clustered around the vesicle, as shown in Figure. (5)[21,22].

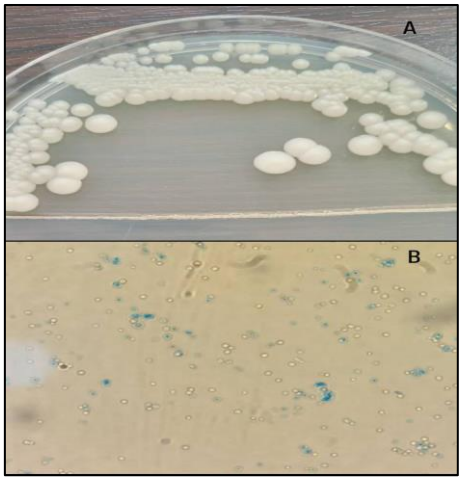

Figure (1): A: *Candida spp*. on SDA, B: Candida spp. observed microscopically at 40× magnification

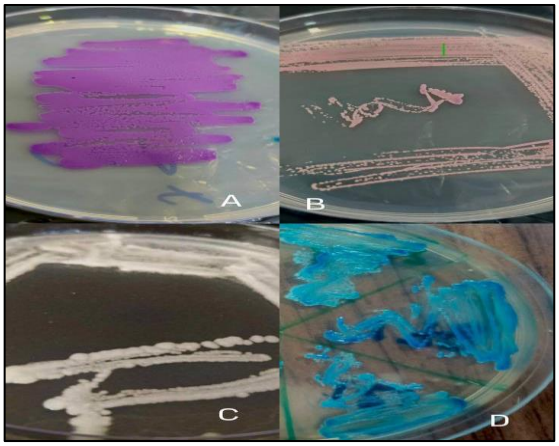

Figure (2): A: *Candida krusei*, B: *Candida glabrata*, C: *Candida parapsilosis*, D: *Candida tropicalis*.

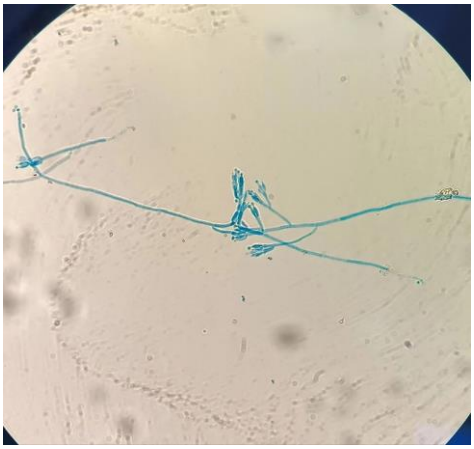

Figure (3): *Penicillium spp*. observed microscopically (40× magnification).

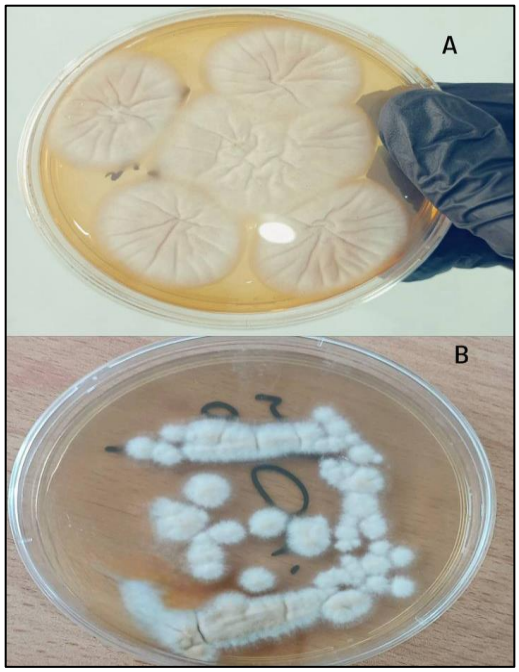

Figure (4): A: *Penicillium citrinum* on Sabouraud Dextrose Agar (SDA). B: *Penicillium chrysogenum* on Sabouraud Dextrose Agar (SDA).

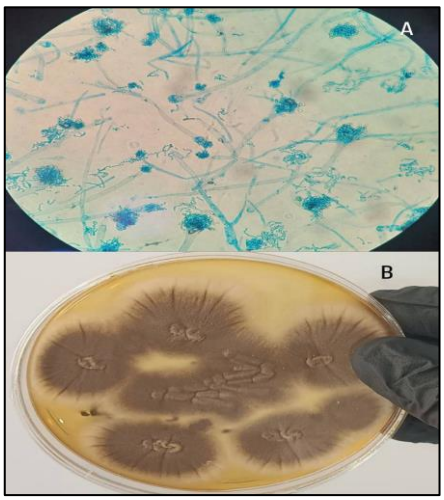

Figure (5): A: *Aspergillus fumigatus* observed microscopically (40× magnification), B: *Aspergillus fumigatus* on Sabouraud Dextrose Agar (SDA).

Phenotypic Phospholipase Activity of *Candida* Isolates from Cold-Food

The phenotypic phospholipase assay performed on *Candida spp*. isolated from cold-food samples showed that all isolates were negative, with no precipitation zone detected on egg-yolk agar, indicating minimal phenotypic expression of this virulence-associated enzyme in food-derived strains. This finding is broadly in line with evidence that non-clinical/environmental (including food-associated) *Candida* isolates often exhibit low or absent phospholipase activity under phenotypic testing conditions [23]. In contrast, clinical studies from mucosal infection sites frequently report appreciable phospholipase positivity among *Candida* isolates (with species-dependent variation), supporting the concept that enzyme expression is strongly influenced by the host niche and infection-related selective pressures rather than food matrices alone [24].

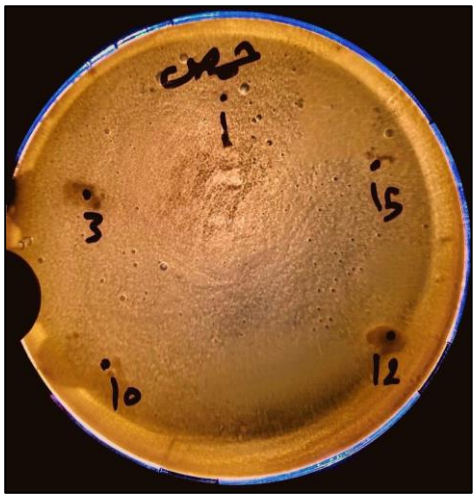

Figure (6): Phospholipase Enzyme Activity of *Candida* Yeasts on Egg Yolk Agar (EYA)

Molecular Identification of Molds

Further, the integrity of extracted DNA from tested mold isolates was also confirmed by molecular analysis (Fig. 7), it can be seen as clear and intact bands after electrophoresis of agarose gel. The resulting DNA was then employed as a template for PCR amplification and amplimers were a unique, approximately 495bp size band which corresponded to the expected size of the targeted gene fragment as displayed in Figure (8). The bands are sharp and clear, with no smearing or distortion, demonstrating that the amplification is efficient and that there are no contaminants or inhibitors. Sequencing demonstrated that the isolates have a high degree of homology with international references in sequence databases. The isolates were closely related to Penicillium chrysogenum, Penicillium citrinum and Aspergillus fumigatus respectively with slight nucleotide polymorphisms that did not affect species demarcation as presented in the table (2).

In general, the result of sequencing supported precise identification for species and could reveal low intraspecific genetic diversity. These nucleotide differences could plausibly reflect natural genetic variation within the species that is not taxonomically meaningful, and may instead be indicative of variability at the strain level, correlating with a strain's adaptive niche [25,26].

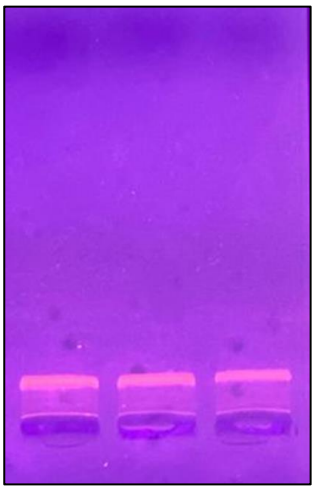

Figure (7): Successful extraction of genomic DNA prior to amplification using polymerase chain reaction (PCR).

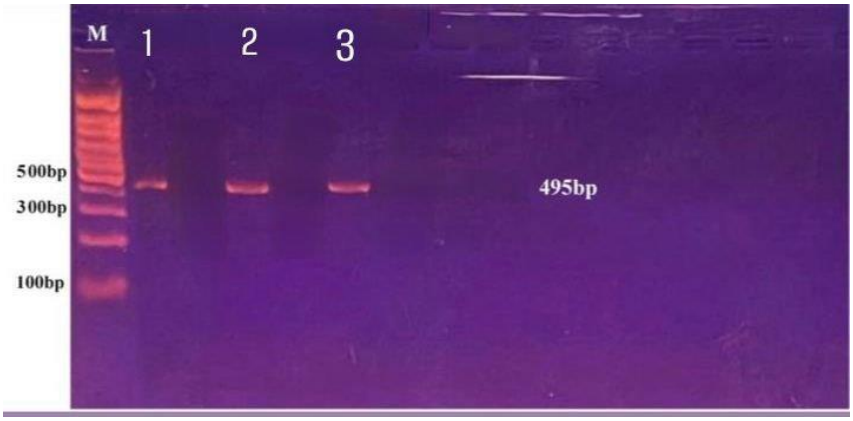

Figure (8): Agarose gel electrophoresis of PCR-amplified beta-tubulin (*BT2*) gene fragment

Table (2): Genetic sequences of molecularly identified fungal isolates

Isolate ID	Genetic Sequence	Fungal Species
Af_Isolate_01	Query 1 CTCGAGCGTGAATGCACCGAGCGTTTCTTCGTAGTTCCTACCAGCTCG AGAGCGGCTTCG 60 Sbjct 210 ..... 269 Query 61 AAGGTTTCCAGCTCCCAAGCCGTCCGCCTCGGCAACATCGGTCAATC CGGTGCCGGTGTC 120 Sbjct 270 ..... 329 Query 121 CAATGCCGTCCGCCTCGGCAACATCGGTCAATCCGGTGCCGGTGTCC AATGCCGTCCGCC 180 Sbjct 330 ..... 389 Query 181 TCGGCAACATCGGTCAATCCGGTGCCGGTGTCCAATGCCGTCCGCCT CGGCAACATCGGT 240 Sbjct 390 ..... 449 Query 241 CAATCCGGTGCCGGTGTCCAATGCCGTCCGCCTCGGCAACATCGGTC AATCCGGTGCCGG 300 Sbjct 450 509	*Penicillium chrysogenum*
Af_Isolate_02	Query 1 CTCGAGCGTGAATGCACCGAGCGTTTCTTCGTAGTTCCTACCAGCTCG .....C.....G.....A..... AGAGCGGCTTCG 60 Sbjct 210 269 Query 61 AAGGTTTCCAGCTCCCAAGCCGTCCGCCTCGGCAACATCGGTCAATC .....T..... CGGTGCCGGTGTC 120 Sbjct 270 329 Query 121 CAATGCCGTCCGCCTCGGCAACATCGGTCAATCCGGTGCCGGTGTCC .....A.....C..... AATGCCGTCCGCC 180 Sbjct 330 389 Query 181 TCGGCAACATCGGTCAATCCGGTGCCGGTGTCCAATGCCGTCCGCCT .....G..... CGGCAACATCGGT 240 Sbjct 390 449 Query 241 CAATCCGGTGCCGGTGTCCAATGCCGTCCGCCTCGGCAACATCGGTC .....A.... AATCCGGTGCCGG 300 Sbjct 450 509	*Penicillium citrinum*
Af_Isolate_03	Query 1 TTCCTCCAAGCCCTCGCACGCTTCCTTCGCAGTAGTTTCTACCAGCTC CAGAGCGGCTTC 60 Sbjct 1250001 ..... 1250060 Query 61 CTTGTTTCCAGCTCCCAAGCCGTCCGCCTCGGCAACATCGGTCAATCC GGTGCCGGTGTC 120 Sbjct 1250061 .....T..... 1250120 Query 121	*Aspergillus fumigatus*

	CAATGCCGTCCGCCTCGGCAACATCGGTCAATCCGGTGCCGGTGTCC AATGCCGTCCGCC 180 Sbjct 1250121 1250180	

Molecular Identification of PLB1 Gene in Candida Isolated from Nasal Swab

Genomic DNA was successfully isolated from all yeast colonies recovered from cold food (HBP) when genotyped by PFGE. The amplified DNA was then used for PCR, indicating the utility of the extraction and viability of the isolates for molecular applications. PCR products were electrophoresed in agarose gels and clear bands were observed for both positive and negative isolates to the PCR assay indicating that both types had been identified. The size of the amplified fragment was around 750 bp commensurate with that of the DNA ladder when compared and could be concluded to be without any smear or distortion validating the integrity and trustworthiness of both PCR reaction products as well as molecular workflow (Figure 9). Detection of the PLB1 gene from cold-food samples varied among Candida species: Candida krusei (Pichia kudriavzevii) had 21 pos- itives out of 26 (67.74%); Candida glabrata presented 6 positive out of 7 (19.35%); only one sample was positive in Candida parapsilosis and two for this same species were negative in the platting method; all three isolates identified as Can- dida tropicalis were positive at molecular test, Before starting our discussion about results obtained we must perform a restriction to readers, since some authors consider Pichia genus member of candidiasis etiologic agents just like Spowitzky and her colleagues [30], while others do not recognize this aspect such Cassone Braunwald [31]. In general, 31/38 (81.58%) yeast isolates tested in the present study were positive for the PLB1 gene, whereas 7/38 (18.42%) were negative because we detected that most of the cold-food yeast isolates carried virulence-associ ated genes such as PLB1 (Table 3).

This seeming discrepancy—isolation of PLB1 (phospholipase B) genes by PCR but a 100% negative phenotypic phospholipase assay—is biologically reasonable, because, while PCR detects genotypal carriage, it does not reflect active expression or the secretion of the enzyme. In *Candida*, phospholipase production is a regulated virulence trait that can be switched on mainly under conditions that mimic host interaction, tissue contact, invasion, or specific environmental cues, whereas isolates originating from cold-food matrices are more consistent with contamination/colonization rather than infection; in such non-host niches, there is often little selective pressure to express invasive enzymes even if the genes are present [27,28].

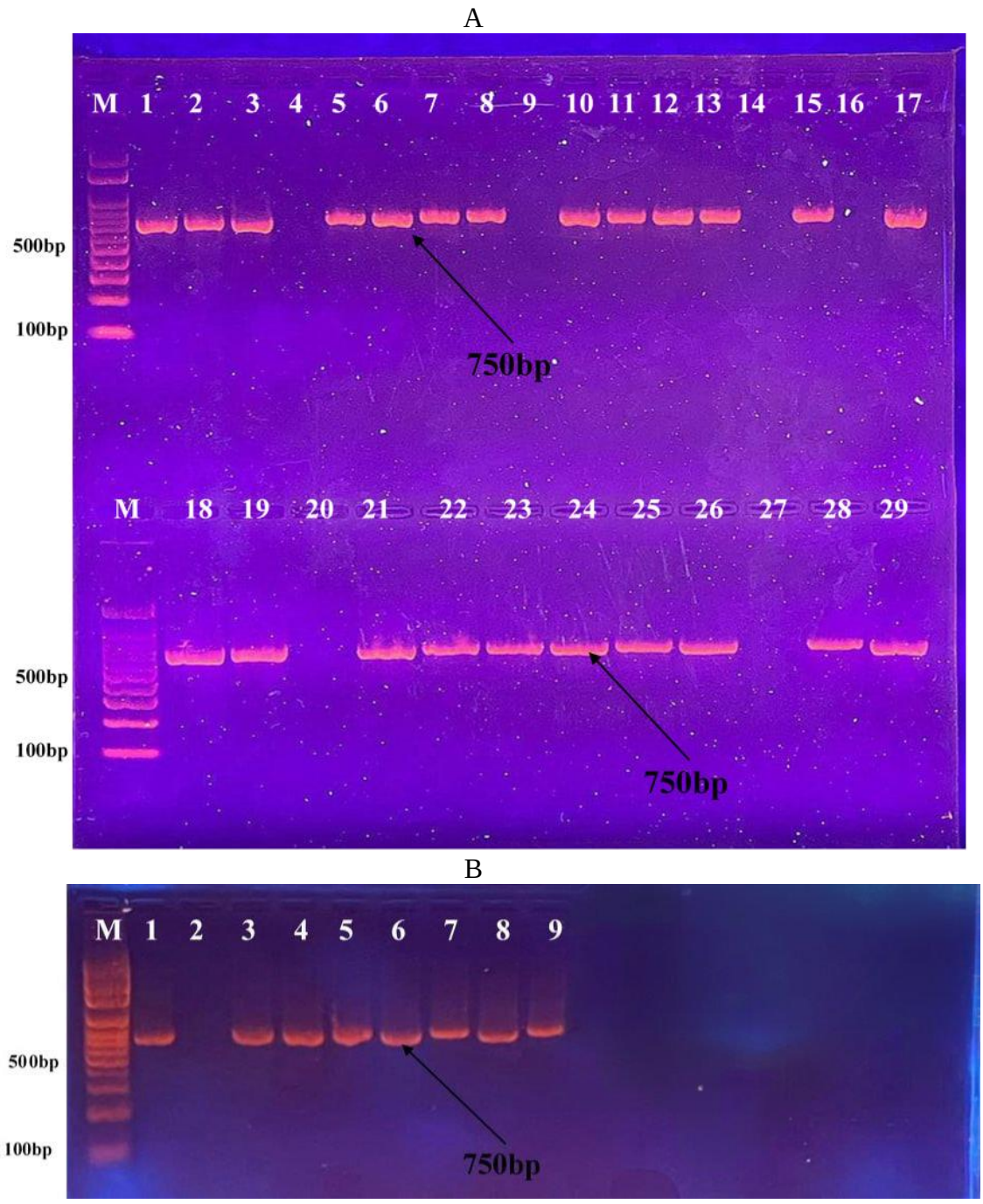

Figure (9) A and B: Agarose gel electrophoresis of PCR-amplified phospholipase B1 (*PLB1*) gene fragment

Table (3): Molecular detection results of the PLB1 gene in yeast isolates from cold-food

Candida spp.	No. of Positive *PLB1* gene	No. of Negative *PLB1* gene
C. krusei (Pichia kudriavzevii)	21 (67.74%)	5 (71.43%)
C. glabrata	6 (19.35%)	1 (14.29%)
C. parapsilosis	(%3.23) 1	(%14.29) 1
Candida tropicalis	(%9.68) 3	(%0.00) 0

Conclusions

In context of the present study, cold-food products served at restaurants can act as a significant source for fungal contamination, where yeasts dominate the community and C. krusei (Pichia kudriavzevii) emerged as the most abundant species demonstrating strong ecological fitness in relation to cold-food processing and storage environments. Mixed fungal growth in multiple samples would also suggest repeated or multiple contamination events, supporting cross-contamination during preparation and/or storage rather than a point source. Even if only occasional molds were recovered, fungal detection is important for food safety and potential health hazards due to the presence of immunocompromised individuals, and molecular identification increased species-level resolution, particularly for low frequency filamentous isolates; in this context, β-tubulin PCR/sequencing consistently identified Penicillium chrysogenum, Penicillium citrinum and Aspergillus fumigatus showing little intra-species variation, reinforcing sequence-based typing as an approach providing definitive ID. Equally essential is the overall negatively uniform phenotypic phospholipase profiles, with one exception, that imply in practice minimal expression of this virulence attribute by food-derived Candida under the employed in vitro conditions; congruently more of contaminators/colonizers than invasive agents. However, the widespread occurrence of PLB1 among yeast isolates suggests that a significant number of food-related Candida strains continue to harbor virulence-related genetic determinants, suggesting pathogenic potential under host-like permissive conditions even if their phenotypic expression is not observed in a food context. In conclusion, discrepancies in genes detected and phenotypic negativity indicate that virulence should be assessed with molecular as well as functional methodology to separate genetic potential from condition-dependent performance, while results also stress the value of intensified hygiene measures and handling procedures during cold-food production at restaurants to minimize yeast contamination and transmission of opportunistic Candida spp.

Conflict of interest

There are no conflicts of interest.

Funding source

The authors have not obtained any source of fundin

Data Sharing Statement

All data are available upon reasonable request to the corresponding author

Reference

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