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Survival and Growth of Food Borne Harmful Fungi during Storage of Vietnamese Ramie Leaf Rice Cake

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ABSTRACT

Background: Ramie leaf rice cake is a traditional dessert in Vietnam. To date, there are no data regarding the nature of mycotoxins that may contaminate this product in this country. This study was conducted to find out if harmful fungal strains were present in traditional ramie leaf rice cake in Binh Dinh province, Vietnam.

Method: The ramie leaf rice cake samples were collected at traditional cake production facilities in Binh Dinh province, transported to the laboratoryand then kept at room temperature. After 3 to 5 days, the cake began to mold, with a slimy surface and a rancid smell. We then carried out microbial analyses. The genetic correlation between the fungal strains discovered was then determined.

Results: The isolation process and preliminary identification through morphological characteristics revealed the presence of four harmful fungal strains. After PCR identification and ITS sequencing, the results showed that the four fungal strains belong to the genus Aspergillus: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus tamarii*and *Aspergillus fumigatus*. The traditional Binh Dinh ramie leaf rice cake in Vietnam is very perishable due to Aspergillus fungus if not consumed quickly. Producers and consumers should pay attention to storage methods, especially during transportation to other areas.

Key words: Aflatoxin, *Aspergillus*, Fungal contamination, Mycotoxin, Rice cake.

INTRODUCTION

Ramie leaf rice cake is one of the traditional desserts in Binh Dinh province, Vietnam. Binh Dinh ramie leaf rice cake is very special because the dough is made with (glutinous) rice flour mixed with pureed ramie leaves. Ramie plant (*Boehmeria nivea*) is a plant that produce fibers. Ramie leaves are green, but when they are cooked (steamed), they turn black. The filling is made from sugar, coconut and green beans. The ramie leaf rice cake has unique ramie fragranceand is soft and delicious compared with other wheat-based baked food.

Binh Dinh ramie leaf rice cake is mainly handmade, on a household scale. Cakes can be steamed. Freshly steamed ramie leaf rice cakes are soft and elastic, but over time they become hard due to retrogradation (Wang *et al.,* 2016) which is at its maximum at 5°C. Therefore, they should not be stored in the refrigerator (Morris, 1990). However, this poses a problem since ramie leaf rice cake can be considered as a good medium for microbial growth because of its rich nutrient content, high water activity and almost neutral pH. Besides, the cake is handmade in the hot and humid climate of Binh Dinh province, Vietnam, so it can rot easily. People working in bakeries struggle to preserve it. Under normal conditions, the cake begins to have filamentous fungus growing on the outside of the shell after only 3 to 5 days.

Fungal infections are the leading cause of post-harvest food spoilage during distribution and storage. Food contaminated with molds not only loses quality and causes economic losses, but also poses a number of health risks to consumers by producing toxins that cause food poisoning (Benedict *et al.,* 2016). Mycotoxins are a major contaminant of foods such as corn, wheat, rice and many others (Marroquin-Cardona *et al.,* 2014). The health consequences of mycotoxins include acute poisoning, cancer, liver disease and neural tube defects (Marroquin-Cardona *et al.,* 2014). Certain fungi, such as *Alternaria* sp., *Aspergillus* sp., *Candida* sp., *Fusarium* sp. (Tomsikova, 2002) and *Penicillium* sp., *Aspergillus* sp. (*A. fumigatus, A. niger, A. terreus*), *Cladosporium* sp., *Alternaria* sp., *Acremonium* sp., *Geotrichum* sp. (Brenier-Pinchart *et al.,* 2006) have been known to commonly appear to spoil food and secrete toxins. In this paper, the research team conducted a study to identify the fungi causing the spoilage of Binh Dinh ramie leaf rice cake during storage in Vietnam.

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MATERIALS AND METHODS

Sample collection and pathogen isolation

Thirty freshly steamed rice cakes were collected at three traditional cake production facilities in Binh Dinh province, transported to the laboratoryand then kept in dry conditions, at room temperature. After 3 to 5 days, the cake appeared moldy, was viscous on the surfaceand had a rancid smell. We then conducted microbial analysis on all mouldy cakes. The cake with symptoms of spoilage was cut into small pieces (about 5 mm in diameter) with a sterile scalpel. The small pieces were transferred to potato dextrose agar (PDA) which contains (g/L): peeled potato 100 g, glucose 20 g, agar 15 g, water 1000 ml and incubated at 28±1°C for 48 h. The mycelia were collected from the edge of the colony and transferred to new PDA plates for purification. The previous step was repeated twice and single colonies of each pathogen were isolated and stored at -20°C for further identification.

Morphological identification of pathogens

The colonies isolated from the third generation of isolated pathogens were subcultured on the new PDA plates and incubated at 30°C for 3 days. The morphological characteristics of the colonies were observed every day. The morphology of the spores and hyphae was observed under an optical microscope. The morphological characteristics were compared with those observed in previous studies to identify the pathogens (Quaglia *et al.,* 2020; Samson *et al.,* 2014; Houbraken *et al.,* 2014; Ranjbar *et al.,* 2019; Samson *et al.,* 2004; Zakaria *et al.,* 2021; Hong *et al.,* 2005).

DNA extraction

Extraction of DNA from fungi was performed from conidial suspensions of isolates in Sabouraud dextrose broth medium (SDB) (Sigma-Aldrich) incubated for 2 days under agitation at 28°C. The resulting mycelium was harvested by filtration.

To extract the DNA, 1 g of mycelium was broken down in the presence of liquid nitrogen. 800 ml DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 1% SDS, 25 mM NaCl) was then added and after incubation at 65°C for 20 min, purified with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). DNA was precipitated by adding two volumes of ice-cold ethanol and 10% 3 M sodium acetate. The precipitate was collected by centrifugation, washed with 70% ethanoland dried. The pellet was resuspended in 1X TE. DNA concentration was determined spectro photometrically at 260 nm (A260) absorption using Nano Drop-1000 (Thermo Scientific).

DNA amplification and sequencing

Primers ITS1 (forward): 5'-TCCGTAGGTGAACCTGCGG-3'; ITS4 (reverse): 5'-TCCTCCGCTTATTGATATGC-3' were used to amplify the ITS region of rDNA.

The 50 µl of reaction mixtures contained 10 ng template DNA, 1 µM of each primer, 100 µM of each dNTP, 5 µl 10X PCR buffer, 1.5 mM $MgCl_2$ and 2.5 Units of Taq Polymerase (*In vitro* gen). The mixtures were subjected to the following amplification program: predenaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing at 52°C for 30s, extension at 72°C for 45s with 35 cyclesand extension at 72°C for another 5 min. PCR products were tested on 0.8% agarose gel, then purified using Gene JETTM PCR Purification Kit (Thermo Scientific, USA). Purified products were sequenced by NK Biotek laboratory (Ho Chi Minh city, Vietnam).

Sequence analysis

The sequencing results were analyzed online using the BLAST analysis program on the National Center for Biotechnology Information (NCBI) nucleic acid database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree analysis was conducted using the Neighbor-Joining (NJ) statistical method and the maximum composite likelihood substitution model on MEGA 7.0 (Kumar *et al.,* 2016). The phylogenetic trees were inferred with 1000 bootstrap (BS) replicates.

RESULTS AND DISCUSSION

Symptoms of ramie leaf rice cake after steaming and storing at room temperature

After steaming, ramie leaf rice cakes were transported to the laboratory and stored at room temperature. After 3 to 5 days, a layer of milky white fungus starts to spread on the surface of the cake; after 5 to 7 days, the cake begins to appear rotten and changes color (Fig 1). These symptoms are typical of a fungal disease. They do not only cause aesthetic damage to the cake, but also affects its sensory quality and are potentially harmful to health. The results also show that the fungus grew faster in the samples packed in sealed plastic bags than in those without plastic packaging.

Morphology of the colonies

Through this investigation at room temperature, four fungal species were isolated from 30 samples of mouldy rice cake collected at different bakery production in Binh Đinh (Fig 2).

CB1: colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidia are globose, dark brown to black and rough-walled.

CB2: Colonies are granular, flat, often with radial grooves. Colonies are white at first but quickly become bright to dark yellow-green, forming concentric circles, dark yellow-green inner circles, followed by light yellow-green circles with white mycelia at the edges. Conidia are globose to subglobose, pale green.

CB3: On PDA the colonies are military olive colour at the center and lighter greenish yellow on the outer ring with white mycelia at the edge and formed sporulation rings; the conidia globose are rough, have thick, rough walls.

CB4: Colonies are typically blue-green with a suede-like surface consisting of a dense felt of conidiophores. Conidial heads are typically columnar. Conidia are produced in basipetal succession forming long chains and are globose, green and finely roughened.

Fungal growth rate

In order to find out the growth rate of the isolated fungi, the study monitored the size of the fungal colonies on a plate of PDA medium at 30°C for 3 days. The results in Table 1 show that the CB4 strain had the fastest growth rate: the diameter of the fungal colony reached 52.8 mm after 3 days. The CB1 strain had a slowest growth rate and, after 3 days, the diameter of the fungal colonies reached 36.3 mm. The CB2 and CB3 had almost the same growth rate and reached 45.6 mm and 41.8 mm respectively.

Identification of fungal strains by ITS gene sequence analysis

The results of ITS1/ITS4 gene sequencing of the fungal strain after analysis were compared with the homologous nucleotide sequences on Genbank using the BLAST SEARCH program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results are shown in Table 2.

Table 2 shows that 4 fungal strains have been sequenced with ITS gene segments and compared with gene banks on NCBI. The harmful fungal strains identified

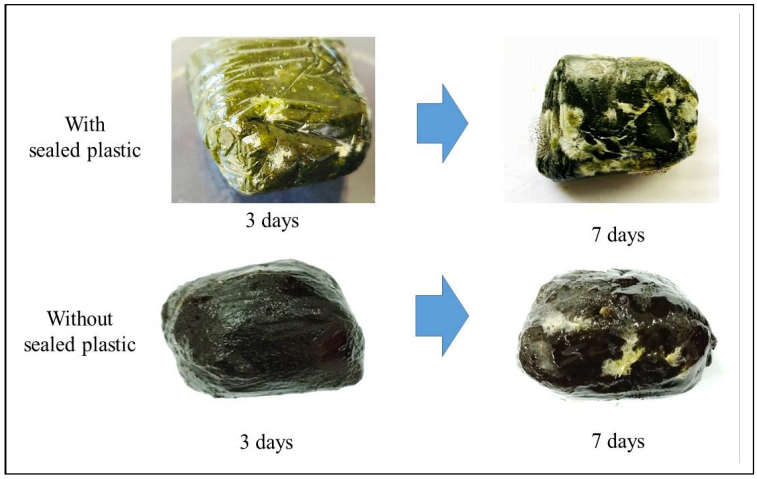

Fig 1: Symptoms of ramie leaf rice cake after steaming and storage at room temperature.

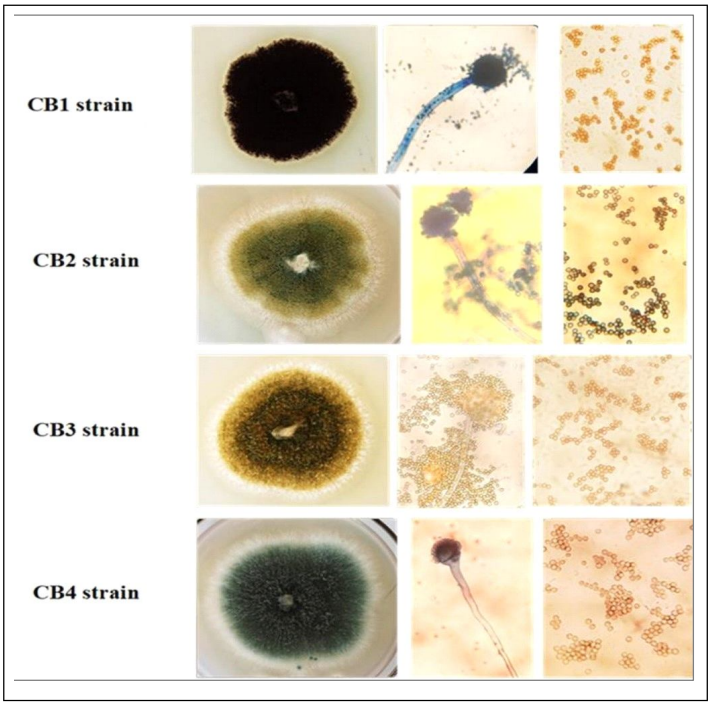

Fig 2: Morphology of colony and colonia of CB1, CB2, CB3 and CB4 on Potato Dextrose agar. Analysis performed at 100x.

at species level are 4 lines belonging to genus *Aspergillus*. It can be seen that the strains CB1, CB2, CB3, CB4 are *Aspergillus niger, Aspergillus flavus, Aspergillus tamarii, Aspergillus fumigatus*, respectively, with gene similarity from 99 to 100%.

Moreover, the phylogenetic tree of *Aspergillus* species was built with the MEGA 7 (Fig 3). This phylogenetic tree has high reliability because the bootstrap rate of all branches is above 50%. According to the phylogenetic tree in Fig 3, the CB1 strain has an ITS sequence almost similar to the sequence of *Aspergillus niger*; the CB2 strain has an ITS sequence almost similar to the sequence of *Aspergillus flavus*; the CB3 strain has an ITS sequence almost similar to the sequence of *Aspergillus tamarii*; and the CB4 strain has an ITS sequence almost similar to the sequence of *Aspergillus fumigatus*.

In addition, in terms of genetic correlation, the 4 fungal strains all belong to the genus *Aspergillus*, so the degree of nucleotide sequence similarity is high (Table 3). However, there are no fungi species that completely overlap with each other, which means that the conservation and variation regions in the rDNA fragment of these fungi still have differences. Strains *Aspergillus tamarii* CB3 and *Aspergillus flavus* CB2 have the least difference with a nucleotide sequence similarity value of 97.62%.

Table 1: Fungal growth rate on PDA medium at 30ºC.

Fungal strains	Colony diameter (mm)		
	1 DAY	2 DAYS	3 DAYS
CB1	16.25±0.15	30.00±0.30	36.25±0.34[a]
CB2	16.75±0.38	33.75±0.28	45.55±0.33[b]
CB3	16.00±0.25	28.75±0.18	41.75±0.38[ab]
CB4	16.50±0.29	34.50±0.23	52.75±0.45[c]

Values followed by different letters within a column are significantly different (P<0.05).

The purpose of this study was to investigate the presence of rot and spoilage fungi in ramie leaf rice cake, a famous dessert in Vietnam. This is a traditional cake found only in Binh Dinh province, a district located in central Vietnam. The climate here is tropical, humid and monsoonal, so pathogenic microorganisms develop easily, which leads to difficulties in preserving food.

According to our observations, all cake samples showed signs of rot, spoilage and mycelium started to appear on the surface of the cake after 3 to 5 days at room temperature. However, some differences in disease status could be observed between cakes, such as time of disease appearance, color of fungi, dry or wet crust, presence of a foul odor or not, *etc*. All of which may be due to the composition of the rice flour, the preparation process, or the storage conditions after steaming. Each ingredient used in these products represents a distinct source of contamination; and mixtures of these components can be an additional source of contamination. Water is an important basic element in food because it provides information about the ability of microorganisms to grow on the surface (Van den Berg, 1984; Sandulachi, 2012). Each ingredient in the rice cake displaying a different water activity, that of the final mixture is unstable, which leads to different symptoms

Table 2: Comparison of gene sequences of fungal strains isolated on NCBI.

Fungi strain	Comparative species on Genbank	GenBank code	Similarity (%)
CB1 strain	*Aspergillus niger strain DTO 390-I7*	MN788116.1	100
	Aspergillus niger strain DTO 391-A1	MN788114.1	100
	Aspergillus niger strain 1901NT-1.4.4	MN585763.1	100
	Aspergillus niger strain 01NT.1.5.4	MH095994.1	100
	Aspergillus welwitschiae strain CBS 139.54b	OL711714.1	100
CB2 strain	*Aspergillus flavus strain A1 chromosome 7*	CP051065.1	100
	Aspergillus pipericola isolate DTO 228-H4	MG662385.1	100
	Aspergillus oryzae strain KBP3	CP031432.1	100
	Aspergillus flavus isolate F4	JF951750.1	100
	Aspergillus flavus culture-collection MUM:10.206	HQ340106.1	100
	Aspergillus flavus isolate DTO 067-I8	MH279385.1	99.89
CB3 strain	*Aspergillus tamarii isolate DTO 364-E3*	MH279435.1	100
	Aspergillus tamarii isolate DTO 213-H5	MG662403.1	100
	Aspergillus tamarii culture-collection NRRL:427	HQ340111.1	100
	Aspergillus tamarii isolate DTO 389-C7	MH279452.1	100
	Aspergillus tamarii strain 122 1 B2	KP784375.1	99.89
	Aspergillus tamarii isolate DTO 145-C3	MH279382.1	100
CB4 strain	*Aspergillus fumigatus strain DTO 402-H1*	MT316338.1	100
	Aspergillus fumigatus strain F7	KR023997.1	100
	Aspergillus fumigatus strain IAL7398	OM259229.1	100
	Aspergillus fumigatus	KM491894.1	99.89
	Aspergillus fumigatus strain IAL7399	OM259228.1	99.89

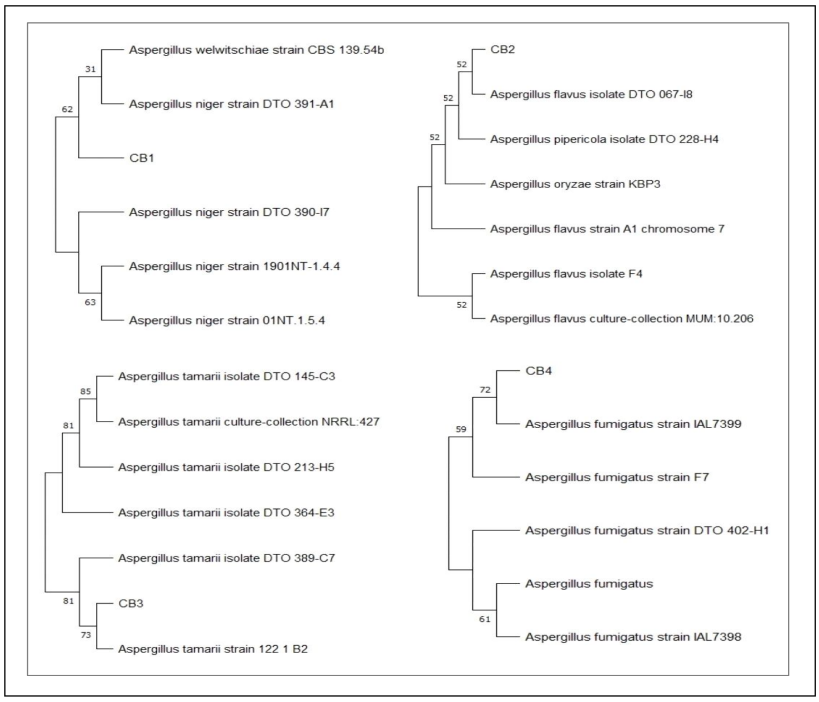

Fig 3: Maximum likelihood pedigree of harmful fungal strains on Ramie leaf rice cake.

Table 3: The degree of nucleotide sequence similarity between harmful fungal strains on ramie leaf rice cake.

Strain	CB1	CB2	CB3	CB4
CB1	100.00	89.59	90.07	89.82
CB2		100.00	97.62	88.65
CB3			100.00	88.44
CB4				100.00

appearing on the cake crust and makes it hard to find a suitable preservation procedure (Iglesias and Chirife, 1976). In this study, we also found that samples packed in sealed plastic bags developed fungus faster than cakes without plastic packaging. This shows that preservation by packaging in plastic packaging does not prevent fungal infection. Therefore, it seems that the traditional packaging process with banana leaves should be preferred over the sealed plastic packaging. In the food production process, packaging is a very important stage because it preserves the quality of the food product during storage, transportation and use (Fadiji *et al.,* 2023). Packaging is necessary to protect food from external factors such as contaminants, gaseous constituents, spoilage bacteria, mechanical loads, or physical damage, it helps to extend the shelf life of food products while ensuring quality and safety (Han *et al.,* 2018; Anwar *et al.,* 2018; Fadiji *et al.,* 2023). Many types of packaging aimed at achieving these objectives are currently on the market and traditional cake manufacturers should therefore prioritize them to protect food and prolong storage time.

The results of our analysis of the morphological characteristics, including the colonies, hyphae and spores, suggest that the pathogen is *Aspergillus* sp. These elements combined with the sequencing of the ITS gene segment and its comparison with the gene bank on NCBI, let us identify four fungal strains at species level, all 4 belonging to genus *Aspergillus* (Quaglia *et al.,* 2020; Samson *et al.,* 2014; Houbraken *et al.,* 2014; Ranjbar *et al.,* 2019; Samson *et al.,* 2004; Zakaria *et al.,* 2021; Hong *et al.,* 2005). *Aspergillus* species can cause rot on various types of crops during pre-harvest, after harvest, during processing, handling, storage and marketing (Perrone *et al.,* 2007). *Aspergillus* species are also the ubiquitous fungi that contaminate various food substrates and produce mycotoxins, such as Aflatoxins, ochratoxin A, patulin, citrinin, aflatrem, secalonic acids, cyclopiazonic acid, terrein, sterigmatocystin or gliotoxin. Mycotoxins exhibit a wide range of toxicity to humans and animal models even at nanomolar concentration (Navale *et al.,* 2021). Some species may also produce other toxic secondary metabolites such as cyclopiazonic acid (Uka *et al.,* 2017). Therefore, the consumption of harmful mycotoxins in adulterated food affects human and animal health even in trace amounts. The growth of fungi and the accumulation of mycotoxins in food and feed are influenced by various factors, among which relative humidity and temperature are critical factors during the storage period (Ghali *et al.,* 2010). Therefore, the traditional steamed rice cakes we studied must be consumed within 3 days when stored at normal temperature. Manufacturers and consumers should find ways to preserve them better if they want to store them for a longer period and avoid food poisoning.

CONCLUSION

This study reported for the first time the diversity of *Aspergillus* in ramie leaf rice cake, a famous dessert in Vietnam. This study also demonstrated that they are very perishable if not consumed quickly. They should probably be included in food safety controls, appropriate to local climatic conditions and specifically preserved when transported to another region.

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Conflict of interest

The authors do not have any conflict of interest to declare.

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