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ORIGINAL ARTICLE

Physicochemical Properties and Microbial Storage Stability of Tiri Traditional Iranian Flat Bread

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ABSTRACT

Background: Tiri bread is one of the oldest known bread types in the Middle East. It is single layer, unleavened, soft, and flat traditional bread baked at home. Dry bread has a long shelf life if stored appropriately at room temperature, but a fresh bread gets moldy with off-flavor 3-4 days after baking. This study has assessed physicochemical properties and microbial storage stability of Tiri bread as traditional flat bread in Iran.

Methods: Twenty samples of fresh home-baked Tiri bread were examined for their physicochemical characteristics and shelf life stability at 4°C and 25°C. Their most common spoiling factors, morphological and molecular attributes were investigated. The breads were assumed unhealthy for consumption when the first sign of mold strains appeared.

Results: The thickness (0.4-0.9 mm), water activity (0.82-0.90), moisture content (18.08-24.13%), salt content (1.59-3.24%), pH (5.75-5.95), and total titrable acidity (2.00-2.90 mL;0.1 N NaOH) were determined. The shelf life of fresh Tiri bread was 10 and 4 days at 4°C and 25°C, respectively. The main factors limiting the shelf life of these breads were appearance of mold as well as development of an off-odor. The most common isolated species were *Aspergillus niger* (31.38%), *A. flavus* (16.12%), *A. tubingensis* (15.12%), *A. awamori* (12.10%), *A. ochraceus* (10.14%), and *Penicillium corylophilum* (16.26%).

Conclusion: As some types of fungi produce harmful toxins which may trigger allergic reactions and can cause harmful infections, it is vital to set out principles concerning safety and health during production and storage of these breads to pay attention to the production and storage conditions of Tiri bread to inhibit mold growth.

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Introduction

Bread has been the primary food staple of the world's population throughout the history. Various flat breads have been consumed in the Iranian Plateau, the Middle East and Mediterranean region for centuries. They are relatively thin (thickness of a few millimeters to a few centimeters) and are consumed fresh soft or dried. They are known to be a major source of dietary protein and calories (1-3). Tiri bread is the oldest known traditional flat bread

which is consumed by many people throughout the Middle East (4). It is single layer, thin, unleavened, and flat bread which is easy to chew (5). The name of "Tiri" bread is originated from "Tir", meaning a wooden rod used to roll and make the dough thin and flat. The appearance of Tiri bread is typically round in shape with an approximate diameter of 45 cm and a thickness of 1-2 mm (6). Tiri bread is similar to Yufka and Chapatti breads. Yufka is a thin, circular, and cream-colored Turkish single layer flat bread which is made by unleavened flour. Chapatti is Indian unleavened flat bread made with flour, water, and salt. Pliability, soft texture, light cream-brown color, a slight chewiness, and baked wheat aroma are the desired quality characteristics of Chapatti (1, 5).

This highly versatile bread can be baked at home or be produce in modern fully automatic industrial lines (7). Tiri bread is prepared with flour, water, and salt with no other ingredients. In the traditional style, the Tiri bread is prepared by thoroughly mixing flour in lukewarm water and addition of about 3% table salt (w/w); while continuously kneading the dough by hand, until it reaches the optimal condition in terms of color and texture. Dough is cut into 200 g balls, rolled into a thin layer by a wooden roller (Figure 1A) and then baked (210±5°C) on a disk-shape metal bread pan convex in shape with a diameter around 100 cm called "Saj" (Figure 1B). The usual baking time is about 1.5-2.5 min (7).

The bread is consumed in both fresh (soft) or dry form. Dry Tiri bread has a fairly long shelf life with no sign of mold growth, particularly if kept in a dry and cool place. The soft one though can be consumed only a few days after preparation. It develops foul odor and can be covered by mold. Similarly, the shelf-life of chapatti is 1-3 days and it becomes unfit for consumption because of mold growth, and texture deterioration depending upon storage conditions (8). In Iran and Iraq, there are several industrial bakeries which have mass production and deliver the Tiri bread in the soft form. As stated, the shelf life of these breads in soft form is less than a week. To extend its shelf life, it is necessary to determine the physicochemical and microbial characteristics of the bread.

The water activity (a_w) and moisture content of flat bread promote the growth and multiplication of microorganisms during the storage period. Spoilage of bread by colonization and growth of fungi represents more than 90% of the total microbial microorganisms involved in contamination (9, 10). The most common spoiling fungi isolated from flat breads are Penicillium, Aspergillus, Fusarium, and Rhizopus genera (11). Molds appear due to external contamination of bread after baking. The contamination generally occur based on steps of transportation, cooling, storage, cutting and packaging (12). Fungal spoilage is considered a major cause of economic loss in bread production, which may also be a source of mycotoxins. As a result, identification of mold species contaminating the bread is highly beneficial (13).

Consumer demand for Tiri bread has an increasing trend. However, Tiri bread suffers from having a short storage life because of development of foul odor and molds. Therefore, it seems crucial to propose approaches to extend its shelf life. The first step to overcome this challenge is to learn what microbial variety and which physicochemical alteration are responsible for this problem. To the best of our knowledge, no study has been conducted for a thorough evaluation of true shelf life of Tiri bread, its physicochemical and microbial properties when stored at room and refrigerated temperatures. So, the main objective of this study was to determine these characteristics as well as the shelf life of Tiri bread at room and refrigerated temperatures.

Materials and Methods

Culture media, including plate count agar (PCA), potato dextrose agar (PDA), malt extract agar (MEA), and De Man, Rogosa and Sharpe agar (MRS), and also chemicals were supplied by Merck

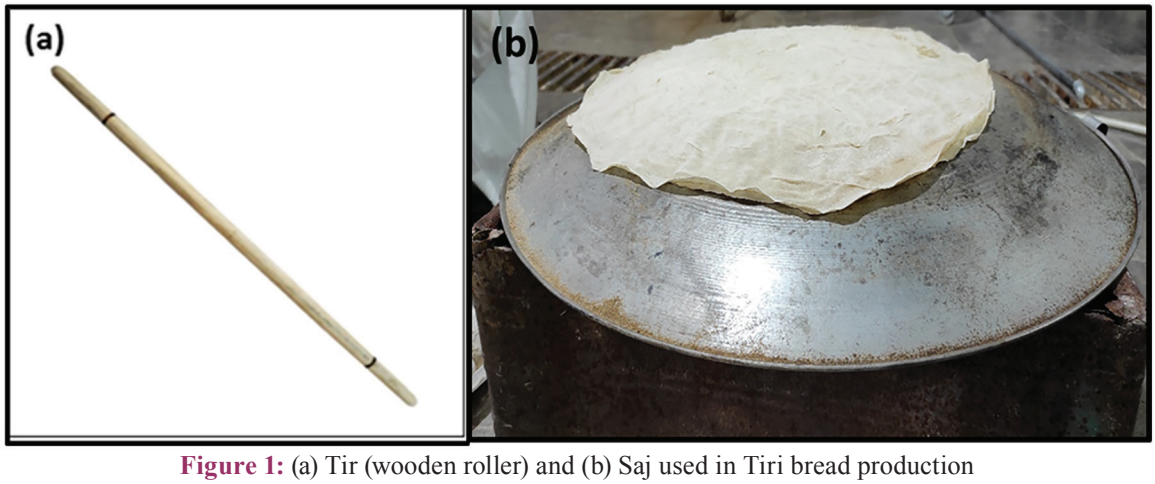

Figure 1: (a) Tir (wooden roller) and (b) Saj used in Tiri bread production

(Darmstadt, Germany). Tiri bread examined in this study was of soft type; while 20 samples of Tiri bread were collected from different regions of Fars province, southern Iran. Bread samples were transferred from baking stage after cooling to polyethylene (PE) bags to perform laboratory tests. The samples were carefully cut into 10 cm × 10 cm pieces and were stored in PE bags at 4˚C and at room temperature (about 25˚C).

Physicochemical properties of Tiri bread samples (weight, diameter, thickness, total titrable acidity, water activity, moisture content, and salt content) were assessed on the baking day. The weight, diameter and thickness were measured. Total titrable acidity (TTA) was measured and expressed as the amount of 0.1 N NaOH in mL (14). The pH was measured using a pH meter (Az86555, Taichung, Taiwan). Water activity (a_w) of bread samples was determined using Aw meter (Novasina AG, lachen, Switzerland) at 25˚C. The moisture content was measured following AACC Official Method 44.01 (15). The salt content was determined using a salinity meter (AZ 86555, Taichung, Taiwan).

By appearance of the first mold colony on the surface of the Tiri bread, its shelf life was terminated (16). Total bacterial, spore, lactic acid bacteria (LAB), and fungal counts were evaluated in all Tiri bread samples every other day. Ten grams of bread sample was homogenized with 90 mL of saline solution for 3 min in a stomacher. Then appropriate dilutions were plated in the media. Total count bacteria were determined on PCA, and the inoculated PCA were incubated at 37˚C for 48 h and then LAB counts were determined on MRS agar medium, with the inoculated plates that were incubated at 37˚C for 48 h (14). Fungal counts were enumerated on PDA medium, and then the plates were incubated at 25˚C for 5 days. For the spore forming bacteria, PCA medium were used to determine the number of spore forming bacteria. First, the dilution of the bread samples was heated to 80˚C for 10 min and then the inoculated plates were incubated at 37˚C for 48 h. Finally, by direct counting of the colonies, the results were presented in Log CFU/g bread.

To isolate the fungi, a block (1 cm × 1 cm) was prepared from the fungus colony formed on the bread surface and placed on PDA medium. Petri dishes were incubated at 25˚C to allow the fungi to grow properly and to purify the fungi, the end-culture method was used. The isolated fungus was removed with sterile scalpel and cultured on water agar medium (WA, 15 g agar, 1000 mL DW). Petri dishes were stored at 25˚C to allow the necessary growth. Depending on the type of fungus after 2 to 3 days, a block was removed from the border of the fungus line with sterile needle under binoculars and added to a new PDA plate; while petri dishes were then incubated at 25˚C (17).

To identify the morphology, colonies of fungi isolated from Tiri bread that belonged to different genera or species of mold were inoculated on the surface of yeast extract sucrose agar medium (YES), Czapek yeast autolysate agar medium (CYA), Czapek yeast autolysate agar with 20% sucrose medium (CY20S), and MEA and were incubated for 7 days at 25˚C. Macroscopic (the rate of colony growth, color, texture and corrugation, and the presence of exudate and pigment) and microscopic (stripes, metulae, phialides, and conidia) characteristics were evaluated using the B-290TB optical microscope and Optika Vision Lite 2.13 analysis software (OPTIKA® Microscopes, Ponteranica, Italy) (18-21).

Nowadays, molecular methods are used in addition to morphological methods to accurately identify all types of fungi. The molecular detection is based on polymerase chain reaction, which has high sensitivity and speed to identify fungi (22). The fungal DNA was extracted from specimens using a genomic DNA extraction kit (Kit DNG™-Plus, SinaClon, Tehran, Iran) according to the manufacturer's instructions. The quantification of extracted DNA was estimated by a NanoDrop spectrophotometer (ND1000 spectrophotometer, Wilmington, DE, USA). DNA of the internal transcribed spacer regions (ITS) of rRNA genes were amplified using the universal primers ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'. Amplifications were performed in a thermal cycler (TC-96/G/H (b) B, China). For each primer set, PCR reactions were performed in 25 µL volumes containing 1 µL of fungal DNA (100 ng μL^{-1}), 1 µL of each primer, and 12.5 µL of PCR Master Mix (CinnaGen, Tehran, Iran) with the remaining volume consisting of ultrapure water. Amplification of ITS region was conducted as follows, pre-denaturation (95˚C, 2 min); 30 cycles: denaturation (95˚C, 30 s), annealing (60˚C, 25 s) and extension (72˚C, 50 s) with a final extension phase (72˚C, 10 min). Successful amplification was confirmed by gel electrophoresis (1 h at 100 V) on 1% agarose gels in Tris-acetate-EDTA buffer. The gels have been moved to the gel doc (gel documentation) system (Syngen, USA) and taken photos of bands (23). At last, amplified products were sent to Cardiogenetic Research Center (Tehran, Iran) for sequencing. All obtained sequences were analyzed and compared with the GenBank database via a BLAST search (NCBI, http://www3.ncbi.nlm.nih.gov) (Bethesda, MD, USA).

The one-way ANOVA was employed to determine the significant differences among the

mean values. Duncan's multiple range test was aimed for comparison of the mean values. The statistical analyses were carried out by SAS software (Statistical Analysis Software, version 9.1; SAS Institute Inc., Cary, NC). A $p<0.05$ was considered statistically significant.

Results

The results of the physicochemical characteristics including salt content, moisture content, a_w and TTA of 20 Tiri bread samples were presented in Table 1. The Tiri bread had a round appearance, cream-color, light weight, and soft texture. The a_w and moisture contents of samples were between 0.82±0.00 and 0.90±0.01 and 18.08±0.05 and 24.13±0.16%, respectively. There were significant differences among salt contents of Tiri bread samples (Between 1.59±0.03 and 3.24±0.03%) and there was a significant difference for TTA of Tiri bread samples (Between 2.00±0.00 and 2.90±0.14). The thickness, diameter, and weight of Tiri bread samples were shown in Table 2. The thickness of samples varied between 0.04±0.01 and 0.09±0.00 mm, while the diameter and weight were between 45.85±0.20 and 49.00±0.14 cm and 85.33±0.34 and 90.76±0.24 g, respectively. Figure 2 demonstrates the pH changes during the storage of 20 Tiri bread samples at 4 and 25°C. Tiri bread samples showed sign of spoilage on day 4 if stored at 25°C. However, no mold growth was observed upon storage up to 10 days after baking if breads were stored at 4°C.

The microbial test (total bacteria, spore, LAB, and fungal counts) results of Tiri bread samples at 4 and 25°C were illustrated in Figure 3. At the beginning of the storage period, the total, spore, LAB, and fungal counts were 2.35-4.76, 0.00-3.33, 0.00-1.44, and 0.00-2.68 Log CFU/g, respectively. Total microbial counts during the storage period in Tiri bread samples increased to 6.13-7.61 and 5.43-7.43 Log CFU/g, at 4 and 25 °C, respectively. Spore forming bacteria counts in Tiri bread samples during the storage period increased to 1.20-2.91 and 2.08-4.54 Log CFU/g, at 4 and 25°C, respectively. LAB counts during the storage period in Tiri bread samples increased to 4.44-6.21 and 3.00-6.46 Log CFU/g, at 4 and 25°C, respectively. Fungal counts of Tiri bread samples during the storage period stored at 4°C and 25°C were 3.79-7.26 and 3.00-7.20 Log CFU/g, respectively. By increasing the storage temperature from 4°C to 25°C, the shelf life of the Tiri bread samples against mold decreased.

Molds on bread appeared when it was stored in a high relative humidity and fairly high temperature. The mold colonies that grew on Tiri bread samples varied from white, yellow to green-gray, and black. In this study, 48 filamentous fungi were isolated from 20 Tiri bread samples and were subjected to phenotypic identification. Fungi isolated from moldy Tiri bread samples, mainly belonged to Aspergillus (84.74%) and Penicillium (16.26%) species.

Table 1: Physicochemical characteristics of Tiri bread samples.

Bread samples	Salt content (%)	*TTA (mL 0.1 N NaOH)	aw	Moisture content (%)
1	2.97±0.04^{b}**	2.05±0.07^{ef}	0.89±0.00^{a}	22.96±0.02^{cd}
2	1.59±0.05^{k}	2.90±0.14^{a}	0.84±0.00^{cd}	18.76±0.29^{g}
3	2.85±0.01^{c}	2.55±0.07^{b}	0.82±0.00^{d}	21.30±0.43^{e}
4	2.58±0.01^{d}	2.10±0.14^{ef}	0.84±0.00^{cd}	18.46±0.34^{g}
5	2.46±0.01^{efgh}	2.00±0.00^{f}	0.89±0.00^{a}	23.38±0.32^{bc}
6	2.50±0.01^{ef}	2.40±0.14^{bc}	0.89±0.00^{a}	24.13±0.16^{a}
7	3.24±0.03^{a}	2.05±0.07^{ef}	0.87±0.00^{bc}	19.47±0.16^{f}
8	2.42±0.01^{fgh}	2.55±0.07^{b}	0.85±0.00^{c}	22.93±0.11^{cd}
9	2.47±0.01^{efg}	2.00±0.00^{f}	0.90±0.00^{a}	23.75±0.27^{ab}
10	2.39±0.02^{gh}	2.05±0.07^{ef}	0.87±0.00^{bc}	18.08±0.09^{g}
11	2.21±0.02^{i}	2.05±0.07^{ef}	0.88±0.01^{b}	22.94±0.10^{cd}
12	1.93±0.02^{j}	2.15±0.21^{def}	0.85±0.01^{c}	19.68±0.18^{f}
13	2.45±0.02^{fgh}	2.15±0.07^{def}	0.83±0.0^{d}	21.13±0.24^{e}
14	1.59±0.03^{k}	2.25±0.07^{cde}	0.86±0.01^{c}	18.36±0.14^{g}
15	2.49±0.03^{ef}	2.45±0.07^{bc}	0.89±0.00^{a}	23.24±0.07^{c}
16	2.54±0.04^{de}	2.05±0.07^{ef}	0.88±0.01^{b}	24.12±0.06^{a}
17	3.18±0.11^{a}	2.15±0.07^{def}	0.88±0.01^{b}	19.37±0.15^{f}
18	2.45±0.03^{fgh}	2.35±0.07^{bcd}	0.88±0.02^{b}	22.53±0.03^{d}
19	2.44±0.03^{fgh}	2.15±0.07^{def}	0.90±0.01^{a}	23.72±0.13^{ab}
20	2.38±0.01^{h}	2.10±0.07^{ef}	0.87±0.01^{bc}	18.08±0.05^{g}

*TTA: Total titrable acidity, **Values are mean±standard deviation of three replicates. In each column means with different superscript letters indicate significant differences ($p<0.05$).

Table 2: Thicknesses, diameters, and weights of Tiri bread samples.

Bread samples	Thickness (mm)	Diameter (mm)	Weight (g)
1	0.050±0.014^{b}*	480.15±0.071^{b}	85.33^{l}
2	0.055±0.007^{ab}	490.00±0.141^{a}	90.76^{a}
3	0.065±0.007^{ab}	460.60±0.141^{def}	90.76^{a}
4	0.045±0.007^{b}	460.50±0.000^{efg}	88.90^{f}
5	0.055±0.007^{ab}	460.80±0.141^{def}	86.72^{j}
6	0.055±0.007^{ab}	470.55±0.495^{c}	87.12^{i}
7	0.065±0.021^{ab}	480.35±0.212^{b}	89.69^{c}
8	0.060±0.014^{ab}	460.75±0.071^{def}	90.51^{b}
9	0.085±0.007^{a}	470.05±0.212^{d}	87.82^{h}
10	0.050±0.014^{b}	460.85±0.354^{def}	89.34^{d}
11	0.045±0.007^{b}	480.15±0.071^{b}	87.92^{g}
12	0.060±0.014^{ab}	490.00±0.141^{a}	85.34^{k}
13	0.070±0.014^{ab}	460.45±0.354^{fg}	97.77^{a}
14	0.065±0.021^{ab}	460.50±0.000^{efg}	88.91^{e}
15	0.075±0.021^{ab}	460.80±0.141^{def}	86.72^{j}
16	0.060±0.014^{ab}	470.00±0.283 ^{de}	87.12^{i}
17	0.065±0.021^{ab}	480.35±0.212^{b}	90.51^{b}
18	0.055±0.007^{ab}	460.75±0.071^{def}	87.82^{h}
19	0.085±0.007^{a}	460.05±0.212^{gh}	89.34^{d}
20	0.090±0.014^{b}	450.85±0.354 ^{h}	87.92^{g}

*Values are mean±standard deviation of three replicates. In each column means with different superscript letters indicate significant differences (p<0.05).

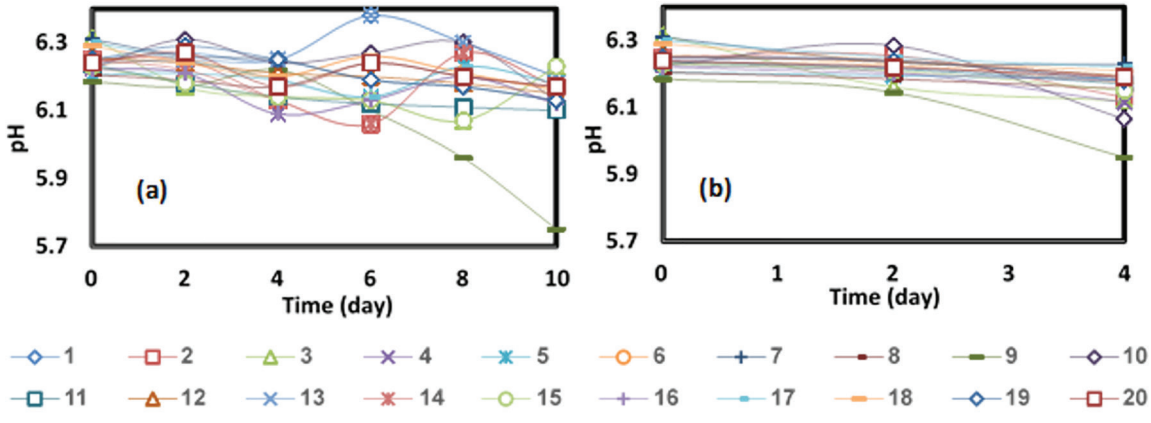

Figure 2: pH changes during the storage of Tiri bread samples at different temperatures of (a) 4 and (b) 25 °C

Table 3: Microscopic features of fungal species isolated from Tiri bread samples.

Property	*A. niger*	*A. awamori*	*A. tubingensis*	*A. ochraceus*	*A. flavus*	**P. corylophilum*
Stipes (µm)	Smooth-wall 456.12±10.32 × 6.04±1.07	Smooth-wall 510.09±9.58 × 7.16±3.02	Smooth-wall 425.24±12.65 × 7.24±2.48	Roughened-wall 285.18±13.68 × 3.02±1.01	Roughened-wall 510.04±14.68 × 11.52±2.41	Smooth-wall 265.26±18.34 × 2.50±1.02
Vesicles (µm)	Subglobose 28.63±1.12	Globose 29.16±3.11	Globose to spherical 36.74±1.49	Globose to elongate 22.56±2.09	Spherical 32.83±3.14	- 4.50±0.50
Metulae (µm)	8.84±1.02 × 3.08±1.17	9.11±1.57 × 3.87±1.66	8.69±2.41 × 2.84±1.06	8.92±2.65 × 3.74±1.34	6.20±1.80 × 2.94±1.01	17.06±2.32 × 2.94±0.34
Phialides (µm)	8.03±0.98 × 2.89±1.03	8.45±1.98 × 2.06±1.01	7.51±2.98 × 2.25±1.09	9.18±1.12 × 2.54±1.06	7.86±2.54 × 2.53±1.08	8.36±0.62 × 2.84±0.38
Conidia (µm)	Prolate to globose 3.31±1.21	Globose 3.24±0.94	Globose 3.88±1.13	Globose to prolate 3.05±0.78	Globose to ellipsoidal 3.21±0.97	Globose to subglobose 2.30±0.22
Sterigmata	Biseriate	Biseriate	Biseriate	Biseriate	Uniseriate	-

*A: Aspergillus **P: Penicillium; Values are mean±standard deviation.

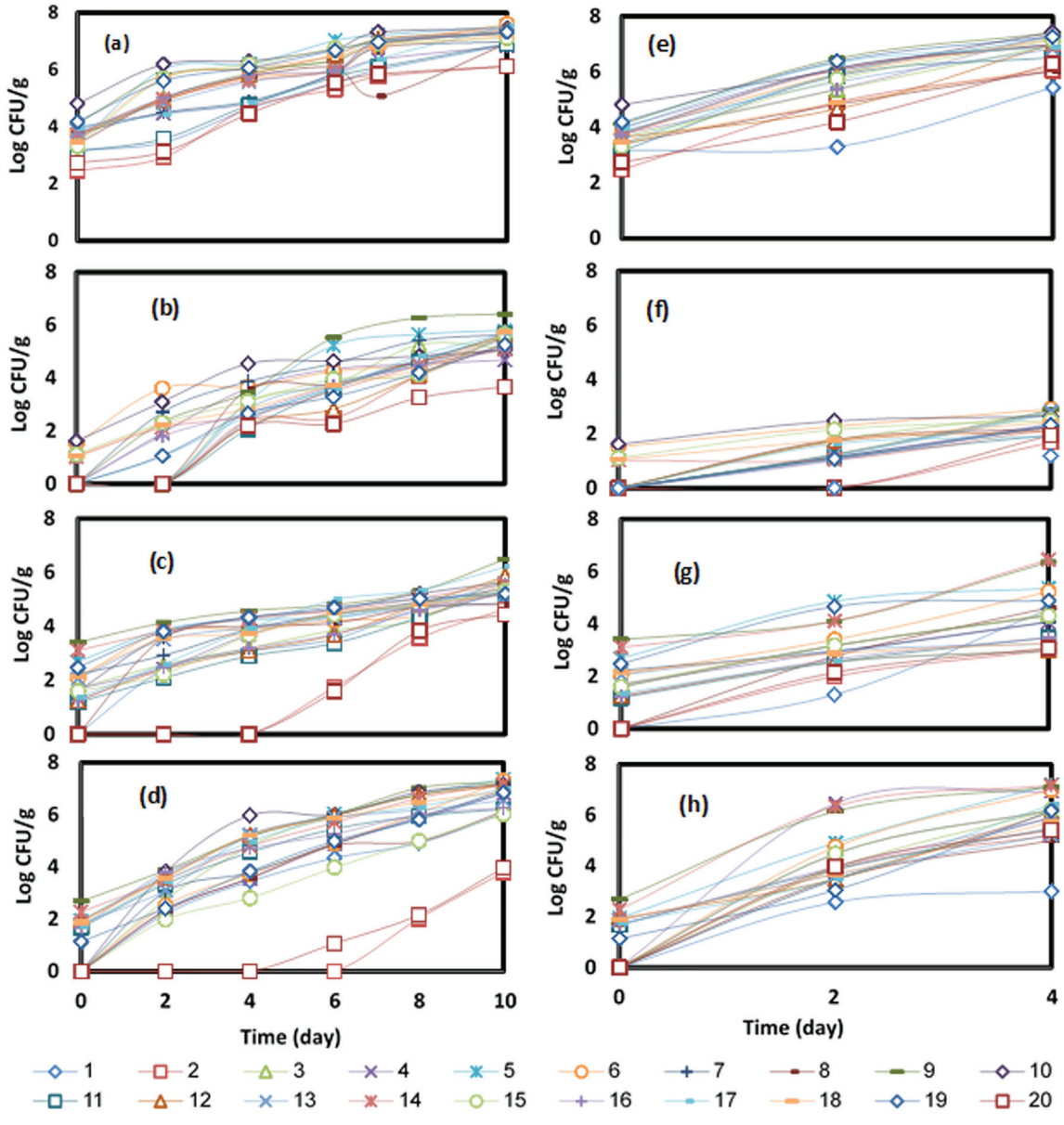

Figure 3: (a,e) total, (b,f) spore, (c,g) lactic acid bacteria, and (d,h) fungi counts of Tiri bread samples stored at 4 (a-d) and 25 °C (e-h), respectively

Table 4: Identification of fungal isolates of ITS region of rRNA gene sequence.

Isolate code number	Species identified	Identity (%)	Length (bp)	Accession number
TBSM1	*A. niger*	100	578	MT588793.1
TBSM2	**A. awamori*	99	575	KR425646.1
TBSM3	A. tubingensis	99	572	MT495451.1
TBSM4	*A. flavus*	100	573	MN893386.1
TBSM5	*A. ochraceus*	99	581	MT582750.1
TBSM6	*P. corylophilum*	100	547	MT892806.1

*A: Aspergillus. P: Penicillium.

In this study, the isolated fungi were examined on the basis of cultural, and microscopic morphological characteristics. Microscopic features of species isolated from Tiri bread were presented in Table 3. The most common species isolated from samples were *Aspergillus niger* (31.38%), *A. flavus* Link (16.12%), *A. tubingensis Mosseray* (15.12%), *A. awamori* Nakaz. (12.10%), *A. ochraceus* G. Wilh. (10.14%), and *Penicillium corylophilum* Dierckx (16.26%). Figure 4 displays the colony morphology of *A. tubingensis*, *A. ochraceus*, *A. niger. A. flavus*, *A. awamori*, and *P. corylophilum*. The molecular identification of mold isolates confirmed the findings of their morphological identification. The ITS region of rDNA sequences was exhibited in Table 4.

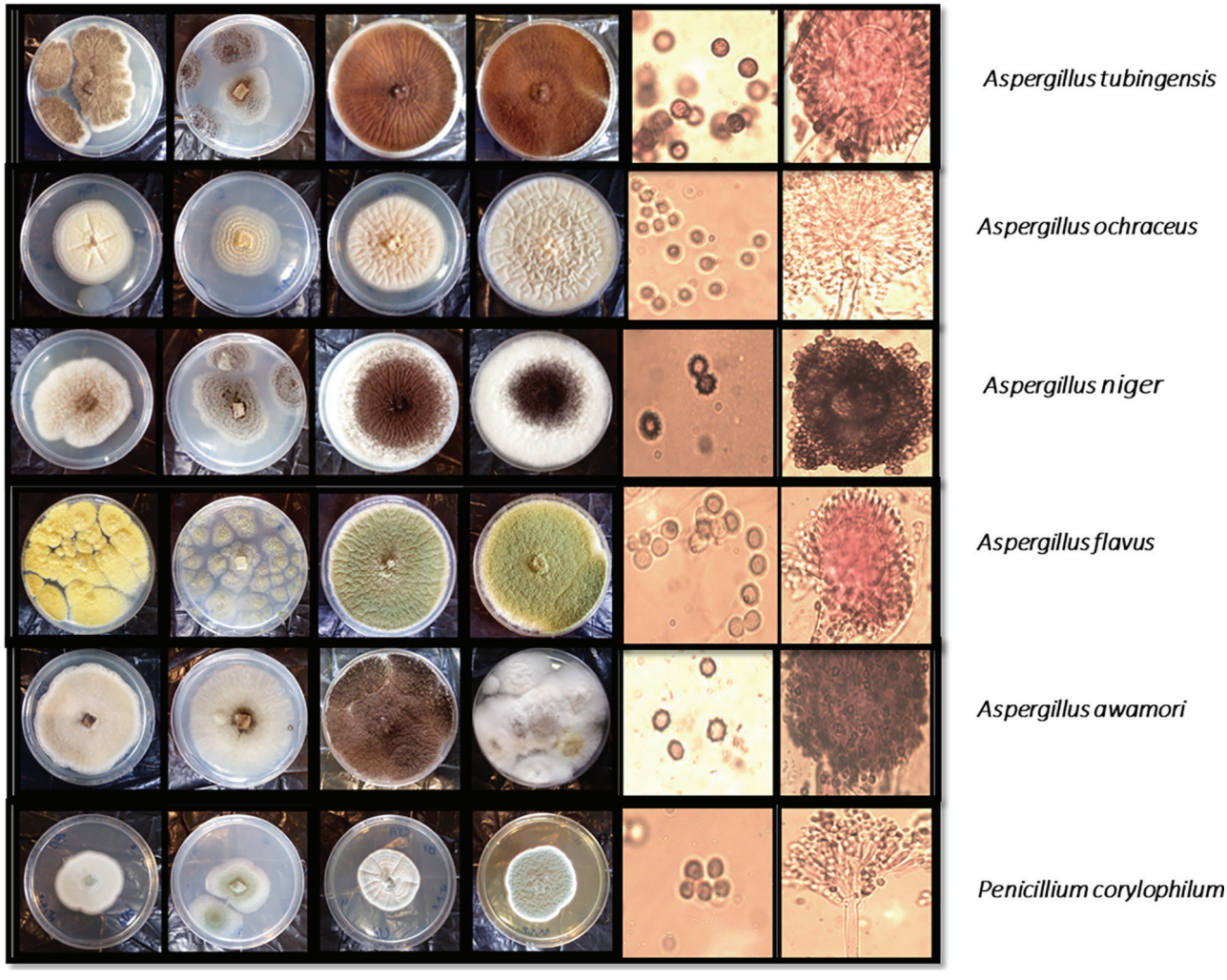

Figure 4: The colony morphology of *Aspergillums tubingensis*, *A. ochraceus*, *A. niger. A. flavus*, *A. awamori*, and *Penicillium corylophilum*; Left to right: cultures on PDA, MEA, CYA, and CY20S (YES for *p. corylophilum*) media, respectively

Sequence BALSTn of the ITS regions of the nuclear encoded rDNA showed significant alignments of 99-100% with the isolated fungal species.

Discussion

Tiri bread is the oldest known traditional bread in the Middle East (5). This study evaluated the physicochemical and microbial properties of traditionally produced Tiri bread samples as well as detection of its spoilage factors. Tiri bread is generally regarded as "thin bread", much thinner than Lavash bread, the most consumed bread in Iran (7). This may be categorized in term of thickness, color and diameter to "Yufka" a Turkish bread with less than 2 mm thickness, around 50 cm diameter with a light cream color. Generally, when discussing flat breads particularly those without leavening, bread is easier to chew if the dough is spread to a very thin layer (6). This thin and large surface bread when is fresh, it can be susceptible to quick spoilage, particularly by mold colonies on the surface. Therefore, it is important to pack and store the bread in a cool and dry condition to achieve a reasonable shelf life.

Water activity is one of the important factors affecting bread shelf life. The a_w has taken the place of moisture as the most useful expression of the availability of water for growth of microorganisms. The a_w of samples were 0.82±0.00- 0.90±0.01. The values are in the range for most baked products (0.7-0.9). Similar to our results, a_w values of other flat breads such as Pita and Taftoon were reported as 088 and 0.89, respectively (24). This high value of water activity clearly makes them likely to get moldy (25). The moisture contents of Tiri bread samples were 18.08±0.05 - 24.13±0.16%. There were significant differences among moisture contents of samples. The 6% variation in moisture content may be attributed to different preparation and baking methods. It is noteworthy that the measured moisture content is within the range (18-25%) expressed in the national standard of Iran (26). Tiri bread requires a very short baking time of 1.5-2.5 min at high temperature (210±5˚C). Short baking time aids in

retaining moisture and softness of Tiri bread. There were significant differences among salt contents of Tiri bread samples (Between 1.59±0.03 and 3.24±0.03%). According to the national standard of Iran (27), the salt content of traditional bread should not exceed 1.8%; while this was not the case for these samples. As stated previously, some samples examined here retained moisture content as much as 3.25%. High salt content helps improving shelf life stability especially when microbiological issues are concerned. Furthermore, presence of salt improves the texture and assists lowering the staling process. The higher salt content of bread lowers the water activity due to the decrease in osmotic pressure, and as a result, its microbial growth is delayed (27). Samapundo *et al.* (2010) investigated the effect of different salt levels (0, 2, 4.2, and 6.4%) on the growth of *P. roqueforti* var. *carneum* and *A. niger* and found that the growth of these molds decreased as the salt level increased from 2% (28).

The pH changes at both temperatures (4 and 25˚C) showed a decreasing trend, but this change occurred more rapidly at 25˚C. There was no significant difference between the pH value of bread samples at 4°C (5.75-6.39) and 25°C (5.95-6.31) at the end of the storage period. According to the national standard of Iran (26), the pH value of traditional bread is considered to be in the range from 5 to 6. In the present study, most samples possessed pH value within the standard range. The national permissible limits of total microorganisms and molds in bread were 5 and 3 Log CFU/g, respectively (29). All examined bread samples were within the standard range. The significant difference (p<0.05) in the total microbial count in bread samples may be due to the differences in storage temperature, moisture content, and a_w of breads, which are factors influencing microbial growth (30).

By increasing the storage temperature from 4 to 25˚C, the shelf life of the Tiri bread samples against mold decreased. At 25˚C, due to the higher temperature and accumulation of moisture on surface of the Tiri bread samples, the condition for activation of mold spores was provided, and the bread samples became moldy in a shorter time. Although the bread is still soft and in numerous cases, the bread color is intact; it is probably unsafe to consume it as mold colonies appear. Similar to our results, Sattari *et al.* (2010) showed that by increasing the storage temperature of pan bread from 5˚C to 25˚C and from 25˚C to 35˚C, the total count of microorganisms and molds increased. So, the highest total count of microorganisms and molds was observed at 35˚C. Also, they found that the shelf life of pan bread at 5°C was longer than at 25˚C and/or 35˚C (31).

In Iran, these breads are sold in a transparent polyethylene packaging at room temperature. One option for producer is to encourage or force the shop owners to store these breads in the refrigerated condition and to observe the product cold chain. Alpers *et al.* (2021) reported that the storage temperature was a factor influencing the microbial shelf life of the bread. Reducing the storage temperature to the refrigerated temperatures decelerated the growth rate of fungi (32). Fungi isolated from moldy Tiri bread samples, mainly belonged to Aspergillus (84.74%) and Penicillium (16.26%) species. Similarly, Garcia *et al.* found that the main genera related to the deterioration of bread samples include Penicillium and Aspergillus species (33, 34). The factors such as a_w range, growth temperature, and tolerance to oxygen tension and preservatives play a decisive role in the predominance of a specific genus and species (30-36). The results obtained in accordance with Axel *et al.* (2017), who presented the most common spoilage organisms found in baked products are *P. corylophilum*, and *A. flavus* (37).

By increasing consumer demand for Tiri bread, it is necessary to find more information about its physicochemical and microbial properties, since these factors determine the shelf life of Tiri bread. The appearance of mold, hence ending the shelf life of Tiri bread is a function of temperature, air humidity as well as moisture content, salt content and pH. Hence, a thorough understanding about influence of these factors is crucial when we want to prevent or delay the fungal growth and possible toxin formation. Usually the mold spoilage of bread is due to post processing contamination. Fresh bread taken off the Saj (bread pan) is usually free of molds or mold spores as the dough/breads is exposed to high temperature while baking. The contamination by spores accumulated in air takes place once the bread is taken off the pan that is continuous during cooling and slicing as well as packaging. The contamination can result into fungal growth which in turn may produce toxic secondary metabolites such as mycotoxins that can cause numerous adverse health issues. Also, these molds produce colored unpleasant spots on the surface and lead to development of bad flavor and odor.

Conclusion

Our findings revealed that the combination of high moisture content and high storage temperature can activate the mold spores as well as their propagation throughout the surface of Tiri bread. The identified fungi of Tiri bread samples were shown to be mostly *A. niger*, *A. flavus*, *A. awamori*, *A. tubingensis*, *A. ochraceus*, and *P. corylophilum* that reminds

the danger of fungal toxins for health status of consumers. So it is necessary to pay attention to the production and storage conditions of Tiri bread to prevent or reduce fungi contamination, especially for toxin-producing fungi.

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Authors' Contribution

SM: formal analysis; investigation; methodology; writing–original draft. MHE: conceptualization; project administration; supervision; validation; writing–review and editing. MN: conceptualization; supervision; validation; writing–review and editing. RMG: conceptualization; supervision; validation; writing–review and editing. MF: formal analysis; methodology; validation.

Conflict of Interest

None declared.

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